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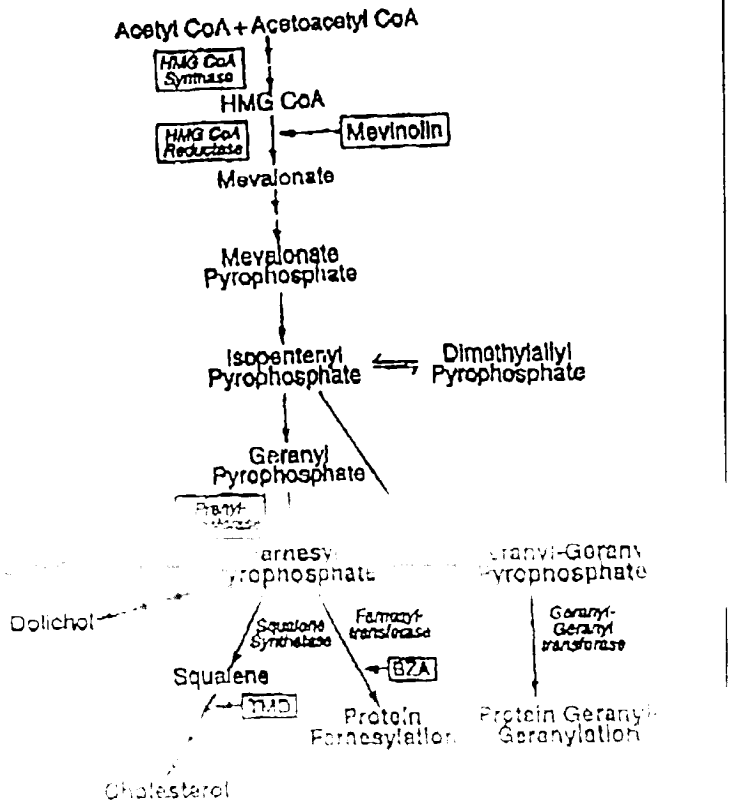
(54) Title: USE OF HMGCoA REDUCTASE INHIBITORS IN THE PREVENTION OF DISEASES WHOSE PATHOGENESIS IS DEPENDENT ON NEOVASCULARIZATION

(57) Abstract

HMGCoA reductase inhibitors have a well–known mechanism in controlling cholesterol metabolism. HMGCoA reductase inhibitors also have a less well–known effect on gene expression. This invention provides a new use for HMGCoA reductase inhibitors in the treatment of diseases whose pathogenesis is dependent on neovascularization. HMGCoA reductase inhibitors are administered at anti–angiogenic therapeutic doses for the treatment of primary and metastatic tumors, inflammatory processes involving new vessel formation, diabetic retinopathy, rheumatoid arthritis, and atherosclerosis. HMGCoA reductase inhibitors affect the expression of genes through interference with the function of small GTP binding proteins (such as Rho). Because of the low incidence of side effects with these agents, HMGCoA reductase inhibitors could also be taken prophylactically to prevent the development of diseases in which the pathogenesis is caused by neovascularization.



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USE OF HMGCoA REDUCTASE INHIBITORS IN THE PREVENTION OF DISEASES WHOSE PATHOGENESIS IS DEPENDENT ON NEOVASCULARIZATION

TECHNICAL FIELD OF THE INVENTION

This invention relates to methods of treating or preventing diseases whose pathogenesis is dependent on neovascularization.

BACKGROUND OF THE INVENTION

Angiogenesis (the formation of new blood vessels from a preexisting vasculature) involves the proliferation, migration, and differentiation of endothelial cells. Growth factors such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF) are potent stimulators of angiogenesis. However, the balance between these pro-angiogenic stimulatory factors and other anti-angiogenic inhibitory factors regulates angiogenesis in the human body. In normal adults, angiogenesis plays a role in the female reproductive system, in the hair cycle, and in wound healing.

Angiogenesis normally occurs in only a few adult human tissues under normal physiological conditions. In the adult, pro-angiogenic stimuli affect the pathogenesis of several disease states, including the growth and development of tumors. New blood vessels might facilitate the inflammation process by bringing in white blood cells and nutrients, and might result in the enhancement of tumor growth. For tumors, the repression or limitation of angiogenic activity could interfere with the development of new tumors and cause the regression of pre-existing tumors. Prevention of angiogenesis could avert the damage caused by the invasion of a new microvascular system. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

For this reason, a considerable interest has arisen in the angiogenic mechanisms of disease and in the discovery of agents which might interfere with angiogenesis (*see,* Folkman, 1 Nature Medicine 27-31 (1995); and Barinaga, 275 Science 482-4 (1997)). A number of anti-angiogenic agents have been developed and agents are undergoing clinical trials. These include (a) antib[illegible]

endothelial cells, interact with the extracellular matrix, and are involved in the differentiation and migration of endothelial cells; (c) molecules which block the activity of

metalloproteinases which breakdown the extracellular matrix and permit the migration of endothelial cells during new vessel formation (such as BB-94 (batimastat; British Biotech Pharmaceuticals, Oxford, UK)); and (d) agents such as angiostatin and endostatin which are secreted by tumors which interfere with the development of metastases by inhibiting new vessel formation (*see,* United States patents 5,885,795 and 5,854,205, both to O'Reilly *et al.*, both incorporated herein by reference). Other anti-angiogenic agents are thalidomide, interleukin 12 (IL-12), TIE-2, anti-tumor necrosis factor α (TNF-α) antibodies, minocycline, α interferon, and the specific angiogenesis inhibitor AGM-1470 (Takeda-Abbott Pharmaceuticals). Anti-angiogenic agents might cause the regression and disappearance of tumors and the stabilization of atherosclerotic plaques (*see,* Moulton *et al.*, 99 Circulation 1726-1732 (1999); Bergers *et al.*, 284(5415) Science 808-812 (1999)). Many of these agents are the subjects of clinical trials, but none have yet been approved for clinical use and their efficacy in human disease is unknown.

What is needed is a method that is known to be safe and which can effectively inhibit the unwanted growth of blood vessels, especially growth of blood vessels into tumors. The method should be able to overcome the activity of endogenous growth factors. The method should also be able to modulate the formation of capillaries in other angiogenic disease states in which angiogenesis plays a role. The method for inhibiting angiogenesis should preferably produce few side effects.

SUMMARY OF THE INVENTION

The invention provides a new use for 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase inhibitors (statins) in the treatment of diseases whose pathogenesis is dependent on neovascularization (angiogenesis). The methods are effective for modulating angiogenesis, and inhibiting unwanted angiogenesis, especially angiogenesis related to tumor growth. Among the new uses of HMGCoA reductase inhibitors are for the treatment and prevention of primary and metastatic tumors, for the treatment and prevention of the inflammatory process involving new vessel formation, for the treatment and prevention of diabetic retinopathy, for the treatment and prevention of rheumatoid arthritis, and for the treatment and prevention of atherosclerosis, by causing the regression of atherosclerotic lesions.

The invention uses HMGCoA reductase inhibitors at therapeutic or prophylactic doses for the treatment or prevention of these diseases. HMGCoA reductase inhibitors are currently

in wide use in the treatment and prevention of coronary artery disease and stroke by reducing the level of lipids in the blood. HMGCoA reductase inhibitors are known to have a low incidence of side effects. Unexpectedly, however, HMGCoA reductase inhibitors can also be used to provide medically important anti-angiogenic effects, through a newly discovered mechanism by which the administration of HMGCoA reductase inhibitors is used to modulate the activity of small GTP-binding proteins, such as Rho. Among the HMGCoA reductase inhibitors that can be used are simvastatin (Zocor®; Merck), pravastatin (Pravachol®; Bristol Myers Squibb), lovastatin (Mevacor®; Merck), atorvastatin (Lipitor;® Park-Davis), fluvastatin (Lescol®; Sandoz) and cerevastatin (Bayer).

The invention also provides a birth control method, in which an effective amount of an HMGCoA reductase inhibitor prevents uterine neovascularization.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effect of simvastatin on the organization of capillary-like structures by human umbilical vein endothelial cells (HUVECs) grown on Matrigel®. FIG. 1A shows a control. FIG. 1B shows the effect of 0.1 μM simvastatin. FIG. 1C shows the effect of 1 μM simvastatin. FIG. 1D shows the effect of 5 μM, simvastatin.

FIG. 2 is a bar graph showing the effect of simvastatin on the proliferation (FIG. 2A) and migration (FIG. 2B) of endothelial cells. Cells were incubated with various concentrations of simvastatin for three days and cells harvested and counted.

FIG. 3 is a bar graph showing the effect of HMGCoA reductase inhibitors in VEGF-mediated angiogenesis in a chorioallantoic membrane (CAM) model. VEGF with and without simvastatin was introduced onto the chorioallantoic membrane in a collagen containing gel sandwiched between a nylon mesh. Placed on the surface of the chorioallantoic membrane. Angiogenesis was quantified by counting the percentage of squares in the top mesh containing blood vessels. Chorioallantoic membranes were incubated with either vehicle, 250 ng VEGF, simvastatin alone, or 250 ng VEGF plus various concentrations of simvastatin.

FIG. 4 shows the effects of simvastatin on FGF-2 stimulated angiogenesis in a mouse corneal pocket model. P denotes the position of the [illegible]

[illegible] FIG. 4B shows angiogenesis stimulated by a polymer containing 10 ng FGF-2 plus 5 μM simvastatin. FIG. 4C - FIG. 4F are photomicrographs of sagittal sections of mouse

corneas. FIG. 4C shows a 24 hr incubation with the polymer alone. FIG. 4D shows a 24 hr incubation with polymer containing 10 ng of FGF-2. FIG. 4E shows a 24 hr incubation with 10 ng of FGF-2 plus 5 μM simvastatin. FIG. 4F shows a 24 hr incubation with 10 ng of FGF-2 plus 10 μM simvastatin.

FIG. 5 is a set of micrographs showing the effects of GGPP, GGTI-287 and C3 exo-toxin on HUVECs cultured on Matrigel, thus demonstrating the involvement of a geranylgeranylated Rho GTPase in the formation of capillary-like structures. (FIG. 5A) control. (FIG. 5B) 5 μM simvastatin plus 10 μM FPP. (FIG. 5C) 5 μM simvastatin plus 10 μM GGPP. (FIG. 5D) 10 μM FTI-277. (FIG. 5E) 10 μM GGTI-287. (FIG. 5F) 5 μg/ml C3 exo-toxin.

FIG. 6 is a schematic representation of the cholesterol biosynthetic pathway, including several cholesterol by-products, such as dolicholphosphate and ubiquinone. FIG. 6 shows the sites of action of BZA, TMD, and HMGCoA reductase inhibitors, such as mevinolin (lovastatin).

DETAILED DESCRIPTION OF THE INVENTION

Introduction.

The invention provides for the use of HMGCoA reductase inhibitors in the treatment and prevention of diseases in whose pathogenesis involves angiogenesis. The mechanism by which HMGCoA reductase inhibitors regulatecholesterol metabolism is well understood. HMGCoA reductase inhibitors also have a less well-known effect on gene expression. But HMGCoA reductase inhibitors also have an effect independent of cholesterol lowering. The non-cholesterol lowering effects of HMGCoA reductase inhibitors are due to the interference of agents with the function of small GTP-binding proteins such as Rho and Ras, which play a role in gene expression. The interference of HMGCoA reductase inhibitors with the function of the small GTP-binding proteins effects the expression of genes coding for growth factor receptors and cytokines. The expression of these genes affect the inflammatory processes, cell migration, and cell cycle regulation involved in atherogenesis and tumor development. Furthermore, these drugs interfere with angiogenesis which is dependent on Rho. Angiogenesis plays an important role in atherogenesis and tumor development. Since these effects involve interference in the farnesylation of Ras or geranylgeranylation of proteins such as Rho or Rho family members, the effects are independent of cholesterol lowering.

This invention thus provides a new use for HMGCoA reductase inhibitors. HMGCoA reductase inhibitors can still be used for the treatment of hypercholesterolemia and secondary prevention in coronary artery disease. Unexpectedly, HMGCoA reductase inhibitors can now be administered to achieve results independent of cholesterol lowering. Based upon this invention, HMGCoA reductase inhibitors can not only achieve plaque reduction, decreased plaque growth, increased plaque stability, and the decreased the likelihood of plaque rupture due to effects on cholesterol lowering, but also by anti-angiogenic effects. The new use of HMGCoA reductase inhibitors is for the treatment of patients with rheumatoid arthritis, diabetes, psoriasis and other inflammatory diseases and both primary and metastatic cancer in which angiogenesis is necessary for the development of the disease. Hence, HMGCoA reductase inhibitors can also prophylactically prevent the development of tumors and the complications of diabetes and the vascularization or atherosclerotic lesions.

The advantages of this invention over existing technological developments are that the prevention of new vessel formation is considered a novel, benign, and curative approach to the treatment of disease. Although anti-TNFα antibody and other anti-proliferative agents have been tested for treatment of rheumatoid arthritis, the HMGCoA reductase inhibitors have far fewer side effects and could be more efficacious than these agents. Furthermore, the method of the invention could, in some cases, replace the chemotherapeutic agents currently used to relieve patients of the devastating side effects of many of these chemotherapeutic agents. Also, the use of antibodies is expensive and often can lead to a reverse immunologic response, thus limiting their use. In the case of diabetic retinopathy, the method of the invention could prevent the development of complications long before the need for laser therapy became necessary. The invention provides a rationale for testing the therapeutic or prophylactic dosage.

HMGCoA reductase inhibitors.

HMGCoA reductase inhibitors exert effects independent of cholesterol lowering. Abnormalities of lipid metabolism are known to importantly affect cardiovascular disease including atherosclerosis and heart failure. 3-Hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors, commonly referred to as "statins" [illegible]

[illegible] enzyme in cholesterol biosynthesis cookistein & BROWN, 343 Nature 425-30 (1990); Grundy, 97 Circulation 1436-9 (1998)). Statins are widely used in the treatment and prevention of

coronary artery and other forms of vascular disease, including hypercholesterolemia and atherosclerotic vascular disease. Many thousand of patients are currently being treated with these agents. The mechanism of action of these agents in the treatment and prevention of coronary artery disease was thought to be due to effects on cholesterol lowering. As a result of clinical trials, which have demonstrated that HMGCoA reductase inhibitors safely reduce cardiovascular morbidity and mortality, HMGCoA reductase inhibitors are now in wide use for the treatment of hypercholesterolemia and atherosclerotic cardiovascular disease (Scandinavian Simvastatin Survival Study Group, 344 Lancet 1383 (1994); Sacks *et al.*, 335 N. Engl. J. Med. 1001-9 (1996), Shepherd *et al.*, 333 N. Engl. J. Med. 1301-7 (1995)). Recently, attention has been focused on non-cholesterol lowering effects of these agents (West of Scotland Coronary Prevention Study Group, 97 Circulation 1440-5 (1998); Sacks *et al.*, 97 Circulation 1446-52 (1998)).

Analysis of clinical data has demonstrated that cholesterol lowering alone does not account for the therapeutic effects of HMGCoA reductase inhibitors (Sacks *et al.*, 97 Circulation 1446-52 (1998), Vaughan *et al.*, 348 Lancet 1079-82 (1996)). Inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors interferes with the synthesis of farnesylpyrophosphate, which is not only a precursor to cholesterol, but is also required for four other pathways (*see*, FIG. 6). These pathways include the biosynthesis of ubiquinone, a component of the mitochondrial oxidative chain; and dolichol phosphate, which is required for the glycosylation of cell surface receptors. Two of these pathways include the farnesylpyrophosphate (FPP) dependent, posttranslational lipidation of small GTP-binding proteins, such as Ras and the GPP-dependent (FIG. 6). Interference with the processes that depend on any one of these four pathways, could be responsible for cholesterol independent effects of HMGCoA reductase inhibitors on atherogenesis and cardiovascular disease (Brown & Goldstein, 21 J. Lipid Res. 505-17 (1980)). Thus, HMGCoA reductase inhibitors might exert effects on the progression of coronary artery disease not only by cholesterol lowering, but also by cholesterol independent mechanisms involving interference with any of these pathways.

Posttranslational lipidation of small GTP binding proteins like Ras and Rho is required for their membrane localization and function. The farnesylation of Ras may be a regulatable process. Induction of the cholesterol metabolic pathway was shown to increase the level of

farnesylation and membrane localization of Ras and the stimulation of Ras dependent gene expression (Gadbut *et al.*, 16 EMBO J. 7250-60 (1997)).

Furthermore, HMGCoA reductase inhibitors interfere with the farnesylation of proteins such as Ras and the geranylgeranylation of proteins such as Rho. Several ligands, receptors, and enzymes involved in cell signaling are either positively or negatively controlled by Rho. Ras dependent TGFβ signaling can be mediated via an effect on the farnesylation of Ras. HMGCoA reductase inhibitors have been shown to increase the Rho-dependent expression of ecNOS, production of NO and inhibition of vascular smooth muscle cell proliferation (Laufs *et al.*, 97 Circ. 1129 (1998); Guijarro *et al.*, 83 Circ.Res. 490 (1998); Laufs & Liao, 273 J. Biol. Chem. 24266 (1998)), effects which might reverse endothelial cell dysfunction and interfere with atherogenesis.

We have shown that TGFβ signaling and the expression of $TGF\beta_1$ and the type II TGFβ receptor are under the negative control of a Rho GTPase. Induction of the cholesterol metabolic pathway decreased the expression of $TGF\beta_1$ and the type II TGFβ receptor, but inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors or the inhibition of the geranylgeranylation of Rho by the geranylgeranyltransferase inhibitor GGTI induced the expression of $TGF\beta_1$ and the type II TGFβ receptor and increased TGF-βsignaling (Park & Galper, 96 Proc. Natl. Acad. Sci. USA 11525-30 (1999)).

Moreover, in the presence of TNFα, the HMGCoA reductase inhibitor lovastatin synergistically decreased the angiogenic response to the intradermal injection of Ras transformed NIH-3T3 cells (Feleszko *et al.*, 81 Int. J. Cancer 560 (1999)).

Angiogenesis affects the pathogenesis of atherosclerosis. Angiogenesis, the formation of new blood vessels from a preexisting vasculature, is physiologically involved in the female reproductive system, in wound healing and in the normal hair cycle (Folkman & Klagsbrun, 235 Science 442-7 (1987)). Angiogenesis also affects the pathogenesis and development of tumors, psoriasis, rheumatoid arthritis and diabetic retinopathy and atherosclerosis (Folkman, 1 Nature Medicine 27-31 (1995)).

Clinical studies have shown an increase in neo-vascularization in atherosclerotic plaques which rupture or develop mural hemorrhage (Pat[illegible] 25 [illegible]

[illegible] vessels with atherosclerotic plaques, these adventitial vessels increase in number and extend into the intima of the atherosclerotic lesions (Barger *et al.*, 310

N. Engl. J. Med. 175-177 (1984)). Casting studies have shown that these intimal vessels are branches of the native adventitial vasa vasorum (Zhang *et al.*, 143 Am. J. Pathol. 164-72 (1993)). Plaque vessels are often found in areas containing large numbers of macrophages, T-cells and mast cells, which can activate angiogenesis (Kaartinen *et al.*, 123 Atherosclerosis 123-31 (1996)). Their close proximity to inflammatory infiltrates means that these vessels may recruit inflammatory cells into the plaques. Furthermore, these vessels may be required for the supply of oxygen and nutrients necessary for the growth of the plaque beyond a certain stage. A recent study using anti-angiogenic agents, endostatin, and TNP-470 (which have no effect on cholesterol levels) shown that they inhibited plaque growth during treatment of cholesterol fed Apo-E -/- mice by 85% and 70%, respectively. Hence, angiogenesis can promote plaque development and inhibition of angiogenesis can suppress plaque growth (Moulton *et al.*, 99 Circulation 1726-32 (1999)).

Others have suggested that HMGCoA reductase inhibitors have anti-angiogenic effects. Treatment of cholesterol-fed monkeys with pravastatin (an HMGCoA reductase inhibitor) resulted in a decrease in both cellularity and neo-vascularization of atherosclerotic plaques (Williams *et al.*, 31 J. Am. Coll. Cardiol. 684-91 (1998)).

Furthermore, treatment of patients with diabetic retinopathy with pravastatin resulted in regression in the vascular lesions (Gordon *et al.*, 112 Am. J. Ophthalmol. 385-91 (1991)).

Vascular endothelial growth factor (VEGF) stimulates angiogenesis during vascular development and in response to pathological stimuli. VEGF affects not only in the development of the vascular system, but also appears to be involved in the pathogenesis of diseases in which angiogenesis has a role. Patients with proliferative diabetic retinopathy contain significantly higher levels of VEGF in their vitreous than those of control patients. These levels exceeded the concentration required for stimulation of proliferation of vascular endothelial cells *in vitro* (Adamis *et al.*, 118 Am. J. Ophthalmol. 445-50 (1994)).

Rheumatoid arthritis is characterized by the proliferation of synovial lining cells, infiltration by inflammatory cells and new blood vessel formation. VEGF is synthesized and released by a large number of the macrophages, fibroblasts and vascular smooth cells in the effected joints (Nagashima *et al.*, 22 J. Rheumatol. 1624-30 (1995)).

Tumor cells also express high levels of VEGF. Clinical trials are in progress to establish the efficacy of anti-angiogenic agents in the treatment of tumor cells. In cardiovascular disease, VEGF has been implicated in both pathologic and therapeutic effects.

Thus, VEGF appears to be up-regulated in artosclerotic arteries and has been implicated in the development of collateral circulation in ischemic myocardium.

Based on the observations in recent clinical studies, VEGF protein and cDNA constructs expressing VEGF have been administered to patients and shown to inhibit intimal thickening following balloon angioplasty and improve blood flow in ischemic limbs. These effects were believed to be mediated through stimulation of endothelial cell growth and angiogenesis respectively (Abedi & Zachary, 272 J. Biol. Chem. 15442-51 (1997)). However, VEGF may also affect the neo-vascularization of atherosclerotic plaques (O'Brien *et al.*, 145 Am. J. Pathol. 883-94 (1994)) and contribute to an increase in atherosclerosis.

VEGF is a dimeric protein with a molecular mass of 45-46 kDa, composed of two 23kDa subunits joined by sulfhydryl bridges. Five isoforms of VEGF, which arise as a result of alternate splicing, have been demonstrated. These isoforms differ in molecular weight and in their ability to bind to cell surface heparan-sulfate proteoglycans and VEGF receptors. VEGF increases vascular permeability, stimulate the expression of proteases required for the breakdown of the basement membranes of blood vessels in the early stages of angiogenesis and initiate cell proliferation and migration (Folkman & Klagsbrun, 235 Science 442-7 (1987)). VEGF also affects the formation of focal adhesions required for cellular proliferation and migration. This effect is mediated via VEGF stimulation of focal adhesion kinase (FAK), a non-receptor kinase, which acts as a scaffold for the assembly of proteins required for the organization of the cytoskeleton and the formation of focal adhesions (Abedi & Zachary, 272 J. Biol. Chem. 15442-51 (1997)).

VEGF receptors are part of a family of tyrosine kinases distinguished by the presence of seven immunoglobulin-like loops in their extracellular domain and a split tyrosine-kinase domain in their intracellular portion (Folkman & Klagsbrun, 235 Science 442-7 (1987)). Two of these receptors, designated VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), are autophosphorylated in response to VEGF binding. The VEGF head to tail homodimer binds to two receptor molecules resulting in receptor dimerization. Ligand binding is followed by autophosphorylation of the receptor which is required for signaling.

There are significant differences between the [illegible]

[illegible] KDR demonstrated that the binding of VEGF to Flk-1/KDR results in the recruitment and phosphorylation of Shc, an SH2-phosphotyrosine-binding domain adapter. Shc recruits

Grb2, another adapter protein containing an SH3 domain which binds Sos, a guanine nucleotide exchange factor for Ras. The activation of Sos results in conversion of Ras to the activated GTP bound state. Similarly, Flk-1/KDR associates with Grb2 and Nck in a ligand dependent fashion (Kroll & Waltenberger, 272 J. Biol. Chem. 32521-7 (1997)). Hence, the activation of Flk-1/KDR stimulated the Ras dependent MAP kinase cascade with the resultant stimulation of cell proliferation. This conclusion is supported by the finding that PD98059, a specific MAP kinase inhibitor, inhibited the effect of VEGF on cell proliferation (Rousseau *et al.*, 15 Oncogene 2169-77 (1997)). Both Flk-1/KDR and Flt-1 stimulate the phosphorylation and activation of p38 kinase (stress activated protein kinase-2). VEGF activation of the p38 kinase pathway stimulates the formation of stress fibers, the assembly of vinculin focal adhesions and cell migration and hence may have an important effect in angiogenesis (Rousseau *et al.*, 15 Oncogene 2169-77 (1997)).

In contrast to Flk-1/KDR, Flt-1 over-expressed in porcine aortic endothelial cells demonstrated only a minimal effect on the activation of MAP-kinase and a very weak phosphorylation of Shc. However, Flt-1 induced the phosphorylation of both phospholipase Cγ and the $p21^{ras}$ GAP p62-p190 complex, which stimulates the GTPase activity of $p21^{ras}$ (Kroll & Waltenberger, 272 J. Biol. Chem. 32521-7 (1997), Seetharam *et al.*, 10 Oncogene 135-47 (1995)). Differences in the function of Flt-1 and Flk-1/KDR have been demonstrated in mice carrying the homozygous disruption in either receptor. Flk-1/KDR knockout mice, which die by embryonic day 8.5, lack endothelial cells and a developing hematopoietic system implicating Flk-1/KDR in the determination of hemato-angioblast progenitor cells and then endothelial cells. This is consistent with the coupling of Flk-1/KDR signaling to MAP-kinase stimulated cell division. In contrast, Flt-1 knockout mice, who also die at day 8.5, have abundant endothelial cells which migrate and proliferate, but do not assemble into tubes and functional vessels (Fong *et al.*, 376 Nature 66-70 (1995)).

Regulation of VEGF expression via hypoxia, growth factors and angiotensin II: VEGF expression is regulated by hypoxia, angiotensin II, thrombin, oncogenes, and cytokines including TGFβ, TNFα, IL-1β, and PDGF.

Both hypoxia and oncogenes regulate VEGF expression at the level of transcription via the stimulation of hypoxia inducible factor (HIF-1). HIF is composed of a β subunit, which is stable under normoxic conditions, and an α subunit which has a half-life of <5 min. Hypoxia markedly inhibits the degradation of HIFα. Studies have shown that in PC12 cells hypoxia

activated two stress activated protein kinases, p38α and p38γ while more prolonged hypoxia activated the Ras dependent p42/44 MAP kinase pathway (Conrad *et al.*, 274 J. Biol. Chem. 23570-6 (1999)). Hypoxia has recently been shown to stimulate the p42/p44 MAP kinase dependent phosphorylation of HIF-1α (Richard *et al.*, 274 J. Biol. Chem. 32631-7 (1999)). Flt-1 and Flk-1/KDR are regulated by hypoxia. While the Flt-1 promoter contains an HIF binding site, no such site has been found for the Flk-1/KDR receptor. VEGF up-regulates Flk-1/KDR gene expression via a feed back loop requiring VEGF binding to the Flk-1/KDR receptor. Since the response of Flt-1 to hypoxia is more immediate than that of Flk-1/KDR, the up-regulation of Flk-1/KDR is secondary to the hypoxic induction of VEGF (Shen *et al.*, 273 J Biol. Chem. 29979-85 (1998)). Inhibitor studies demonstrated that VEGF regulation of Flk-1/KDR expression was dependent on tyrosine phosphorylation, PKC, Src kinase and stimulation of the ERK pathway (Shen *et al.*, 273 J Biol. Chem. 29979-85 (1998)).

Both thrombin and angiotensin II stimulate angiogenesis. Thrombin stimulates angiogenesis in the chick chorioallantoic membrane (CAM) assay. Incubation of HUVECs with thrombin increased the expression of VEGF and sensitized the cells to VEGF stimulation of [^{3}H] thymidine incorporation and cell growth. mRNAs coding for both Flt-1 and Flk-1/KDR were increased and Flk-1/KDR protein was increased by 200% (Tsopanoglou & Maragoudakis, 274 J. Biol. Chem. 23969-76 (1999)). Thrombin signals by the stimulation of the c-Jun N-terminal kinase/ stress activated protein kinase (JNK/SAPK) pathway, the p38 kinase/stress activated protein kinase pathway and the extracellular signal-regulated kinase (ERK) pathway. Inhibitor studies have implicated the ERK pathway and protein kinase C in the regulation of Flt-1 and Flk-1/KDR by thrombin (Tsopanoglou & Maragoudakis, 274 J. Biol. Chem. 23969-76 (1999)).

Angiotensin II induces hypertension, and atherosclerosis *in vivo* (Li *et al.*, 143 Atherosclerosis 315-26 (1999)). Angiotensin II also stimulates angiogenesis and markedly increase the expression of vascular endothelial growth factor (VEGF) in human vascular smooth muscle cells (Williams *et al.*, 25 Hypertension 913-7 (1995)) and angiogenesis, VEGF, Flt-1, and Flk-1 in cultured retinal microcapillary endothelial cells (Otani *et al.*, 82 Circ. Res. 619-28 (1998)). Incubation of retinal microcapillary endothelial cells with angiotensin II potentiates bFGF-stimulated tube formation on a three-dimensional collagen gel (Otani *et al.*, 82 Circ. Res. 619-28 (1998)). Like thrombin, angiotensin II stimulates the ERK,

JNK, and p38 MAP kinase pathways. Angiotensin II-stimulation of KDR expression was shown to be dependent on tyrosine phosphorylation and activation of PKC by PMA.

Effects of blood pressure and angiotensin II in animal models for atherosclerosis. The relationship of hypertension to atherosclerosis has been well established. Studies in a number of animal models have demonstrated that angiotensin II converting enzyme inhibitors or angiotensin II type 1 receptor blockers interfere with the progression of atherosclerosis in hypertensive animals. Recently ApoE-/- mice have been generated which demonstrate profound hypercholesterolemia and the propensity to develop atherosclerotic lesions with similarities to those found in humans. Treatment of ApoE-/- mice with a combination of an angiotensin II type 1 (AT_1) receptor blocker losartan and an α_1-adrenergic receptor blocker prazosin lowered blood pressure and decreased average plaque size by 43% (Makaritsis *et al.*, 32 Hypertension 1044-8 (1998)). In a study in ApoE -/- mice intraperitoneal injection of angiotensin II (0.1 ml of 10^{-7} M each day) for 30 days increased atherosclerotic lesions by 95% compared to placebo mice. Peritoneal macrophages from these animals demonstrated a 90% increase in cholesterol biosynthesis, as measured by incorporation of [^{3}H]-acetate into cholesterol. This effect was reversed by both the angiotensin converting enzyme (ACE) inhibitor fosinopril and losartan. Finally, in a macrophage cell line angiotensin II increased the expression of HMGCoA reductase in a dose dependent manner (Keidar *et al.*, 146 Atherosclerosis.249-57 (1999)). Thus, these data suggest that angiotensin not only increases blood pressure, but also increases the production of cholesterol and the products of the cholesterol pathway. Hence, angiotensin may affect Ras-dependent and Rho-dependent gene expression. These data provide the basis for a new relationship between hypertension and agents (such as statins) that affect cholesterol metabolism which might affect the atherogenic effect of hypertension (as shown in EXAMPLE 6-9).

Evidence for an effect of VEGF and VEGF receptor expression in the neovascularization of atherosclerotic lesions. VEGF is involved in the neovascularization of atherosclerotic lesions. The expression of VEGF and VEGF receptors has been compared in normal and diseased coronary arteries. While the expression of both VEGF and VEGF receptors was undetectable in normal coronary arteries, a correlation was found between the severity of artherosclerotic involvement of vessels and the extent of expression of VEGF, Flt-1, and Flk-1/KDR. Hypercellular and atheromatous lesions showed positive staining for VEGF in endothelial cells, macrophages and smooth muscle cells. Large occlusive lesions

with extensive neovascularization demonstrated intense staining for VEGF, Flt-1 and Flk-1/KDR in macrophages, endothelial cells and microvessels (Inoue *et al.*, 98 Circulation 2108-16 (1998); Chen *et al.*, 19 Arterioscler. Thromb. Vasc. Biol. 131-9 (1999)).

The effect of integrins in VEGF signaling. Angiogenesis involves the proliferation, migration, and differentiation of endothelial cells. Migration requires the formation of stress fibers and the assembly of focal adhesions. Signals from integrin receptors are integrated with those from VEGF signaling to organize the cytoskeleton, form focal adhesions, and stimulate migration (Kumar, 17 Oncogene 1365-73 (1998)).

Integrin receptors are composed of noncovalently associated α and β chains which form heterodimeric receptor complexes. Both subunits contain a large extracellular domain and a cytoplasmic carboxy terminal of variable length. There are 17 α subunits and 8 β subunits which combine to form 22 different receptor complexes. The extracellular domains of the α and β chains form the ligand binding sites. Integrin receptors recognize the sequence RGD in their extracellular matrix ligands. However, integrins can recognize the differences between ligands with a degree of specificity: $\alpha_v\beta_3$ binds to vitronectin, $\alpha_5\beta_1$ binds to fibronectin, and $\alpha_2\beta_1$ binds to collagen and $\alpha_v\beta_5$ binds to laminin (Soldi *et al.*, 18 EMBO J. 882-92 (1999); Giancotti & Ruoslahti, 285 Science 1028-32 (1999)). Integrins not only bind to components of the extracellular matrix, but also bind to soluble ligands such as fibrinogen or to counter-receptors such as the intracellular adhesion molecule (ICAM) on nearby cells. Integrins can be cell type specific. Binding of integrins to the extracellular matrix results in the activation of members of the Rho family of small GTP-binding proteins leading to clustering of integrins, association with cytoskeletal proteins and the binding to molecules, which promote downstream signaling. These aggregates of extra cellular matrix proteins, integrins, and cytoskeletal proteins form focal adhesions where integrins link the outside matrix to the intracellular cytoskeletal complex. Signaling from these focal adhesions regulates cell adhesion, changes in cell shape and cell movement. The cytoplasmic tails of integrins are short and devoid of enzymatic activity. Hence integrins associate with adapter proteins which permit them to interact with the cytoskeleton, cytoplasmic kinases and transmembrane growth factor receptors.

the assembly of other components of focal adhesions. FAK is recruited to the nascent focal adhesions by interacting directly with the tail of the integrin β subunit or indirectly through the

cytoskeletal proteins talin and paxillin. Integrin activation by ligand binding results in autophosphorylation of FAK Tyr^{397}, which generates a site for the binding of the SH2 domain of Src and Fyn. These kinases phosphorylate FAK associated proteins paxillin, tensin, and $p130^{cas}$, which is a docking protein which recruits two adapter proteins, Crk, and Nck (Giancotti & Ruoslahti, 285 Science 1028-32 (1999)).

Crk is an adapter protein with both SH2 and SH3 domains and is capable of activating the JNK pathway. Expression of $p130^{cas}$ (major binding protein for the SH2 domain of Crk) has also been show to activate JNK. Rac (a member of the Rho family of GTPases) is responsible for initiating the activation of JNK pathways. Expression of a dominant negative Rac blocked activation of the JNK pathway by $p130^{cas}$ and Crk. (Dolfi *et al.*, 95 Proc. Natl. Acad. Sci. USA 15394-9 (1998)). Src also phosphorylates FAK Tyr^{925}, creating a site of the binding of the adapter Grb2 which binds Sos (the Ras GTP exchange factor), which is coupled to the activation of Ras and the ERK pathway (Schlaepfer *et al.*, 372 Nature 786-91 (1994)). Finally, FAK has been found to be associated with PI 3-kinase which activates Akt kinase signaling.

Integrins are necessary for optimal activation of VEGF signaling. Thus, cell attachment is required for optimal activation of VEGF receptors. Furthermore, VEGF is a poor activator of JNK and integrin activation potentiates the JNK and MAP kinase signaling stimulated by VEGF. Significant cross-talk has been demonstrated between VEGF and integrin signaling. In cells incubated with VEGF, $\alpha_v\beta_3$ is physically associated with Flk-1/KDR (Soldi *et al.*, 18 EMBO J. 882-92 (1999)). Adhesion to matrix proteins potentiates insulin, PDGF, and FGF stimulated receptor tyrosine autophosphorylation (Soldi *et al.*, 18 EMBO J. 882-92 (1999), Moro *et al.*, 17 EMBO J. 6622-32 (1998), Schneller *et al.*, 16 EMBO J. 5600-7 (1997)). Endothelial cells cultured on vitronectin demonstrated enhanced VEGF-stimulated tyrosine phosphorylation of Flk-1/KDR in the absence of an effect on the expression of the receptor. Furthermore, an anti-β_3 antibody inhibited VEGF-stimulated migration, polarization and proliferation (Soldi *et al.*, 18 EMBO J. 882-92 (1999)). Fibronectin was shown to increase the expression of VEGF in retinal pigmented epithelial cells (Mousa *et al.*, 74 J. Cell Biochem. 135-43 (1999)). Vitronectin, fibronectin, and thrombospondin increase the expression of VEGF in retinal pigmented epithelial cells (Soldi *et al.*, 18 EMBO J. 882-92 (1999)). Thus, integrin activation influences cell cycle progression, cell survival, and gene expression stimulated by VEGF signaling in addition to their effects on cell adhesion and cell

morphology. Conversely, growth factors potentiate integrin signaling. Thus, VEGF stimulates the tyrosine phosphorylation of FAK and paxillin in HUVECs and the endothelial cell line ECV304 (Abedi & Zachary, 272 J. Biol. Chem. 15442-51 (1997)). VEGF increases the expression of α_v and β_3 mRNA and the $\alpha_v\beta_3$ ligand osteopontin (OPN) in dermal microvascular endothelial cells (Senger *et al.*, 149 Am. J. Pathol. 293-305 (1996)). The importance of this cross-talk may be that VEGF stimulation alone or integrin stimulation alone might not be sufficient for the activation of certain pathways. Hence, cross-talk between integrin signaling and VEGF signaling might have an important effect in cellular proliferation and migration.

Effect of Rho family members in angiogenesis and VEGF signaling. Although members of the Rho family of GTPases are known to have an important role in integrin signaling, their effect in angiogenesis and in VEGF signaling is not yet understood (Parsons, 8 Curr. Opin. Cell Biol. 146-52 (1996)). Three members of the Rho family of GTPases have been implicated in integrin signaling: RhoA, Rac, and Cdc42. The interrelationships between these three family members are not well understood. Microinjections of Swiss 3T3 cells have demonstrated that RhoA rapidly stimulated stress fiber and focal adhesion formation (Ridley & Hall, 70 Cell 389-99 (1992)). Cdc42 stimulates actin polymerization to form filopodia, or microspikes. Like RhoA, Rac and Cdc42 stimulate the formation of focal complexes, which contain vinculin, paxillin and FAK, which differ from focal adhesions in both size and their lack of dependence on RhoA (Nobes & Hall, 81 Cell 53-62 (1995)). The activation of Cdc42 sequentially stimulates Rac and then RhoA, so that the formation of filopodia and lamellipodia is coordinately regulated in the control of cellular motility (Nobes & Hall, 81 Cell 53-62 (1995), Mackay & Hall, 273 J. Biol. Chem. 20685-8 (1998)). Stimulation by RhoA in scrape loaded Swiss 3T3 cells or stimulation by lysophosphatidic acid or bombesin in the presence of Cytochalasin D caused the phosphorylation of FAK, p130^{cas} and paxillin in the absence of stress fiber formation demonstrating that the formation of focal adhesions and stress fibers were independent processes (Flinn & Ridley, 109 J. Cell Sci. 1133-41 (1996)). Dominant negative mutants of Rho family members were used to demonstrate that adhesion of Rat-1 cells to fibronectin was independent of Rho family members. However, F-actin levels were phosphotyrosine [illegible] was unaffected by Rac and Cdc42, but after an initial 30 minute lag period was decreased by a dominant negative RhoA mutant and C3 exotoxin. A dominant

negative RhoA mutant also decreased the phosphorylation of paxillin by 50%. Integrin stimulation of ERK2 was inhibited by all three Rho family members in the order Cdc42>RhoA>Rac. However, in PDGF stimulated Rat-1 cells Cdc42 had no effect on ERK2 activation (Clark *et al.*, 142 J Cell Biol. 142:573-86 (1998)). Similarly, PI 3-kinase and Akt kinase were activated by Cdc42 only. Rac and Cdc42 have been implicated in the activation of the JNK/SAPK and p38 MAP kinase cascades in response to stresses such as UV light and osmotic shock. Ras activates Rac. Studies using dominant negative Rac have demonstrated that Rac is necessary for malignant transformation by Ras.

Like Ras, Rho is activated by a large group (>20) of guanine nucleotide release factors (GEFs) and at least 10 Rho GTPase-activating proteins (GAPs). Also a group of guanine nucleotide dissociation inhibitors (GDIs) which act as chaperons of GDP bound Rho from the membrane to the cytoplasm have been found. Rho family members may affect the cross-talk between integrin and growth factor signaling. The carboxy-terminal of FAK is associated with the Rho GAP, designated Graf (GTPase regulator associated with FAK (Hildebrand *et al.*, 16 Mol. Cell Biol. 3169-78 (1996))). Stimulation of PC12 cells by epidermal growth factor resulted in the phosphorylation of Graf providing a mechanism by which growth factors exert a Rho dependent effect on the cytoskeleton (Taylor *et al.*, 273 J. Biol. Chem. 8063-70 (1998)). Immunoprecipitation studies have demonstrated that RhoA might directly effect growth factor signaling by binding directly to the PDGF receptor (Zubiaur *et al.*, 270 J. Biol. Chem. 17221-8 (1995)).

Angiogenesis and Disease.

The invention provides a method for treating diseases and processes that are mediated by angiogenesis. The term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development, and formation of the corpus luteum, endometrium and placenta. The term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels, and blood vessels.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of

blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

A delicate balance between stimulatory and inhibitory factors regulates angiogenesis. Pro-angiogenic stimuli have a critical role in the pathogenesis of several disease states, including inflammatory diseases, and the growth and development of tumors (*see,* Iruela-Arispe, 78 Throm. Haemost. 672-7 (1977)). Vascular endothelial growth factor (VEGF) appears to be the most endothelial cell specific and unequivocal angiogenic factors (*see,* Leung *et al.*, 246 Science 1306-9 (1989)). Basic fibroblast growth factor is another angiogenic cytokine. Thrombospondin 1 is one of a number of anti-angiogenic factors found in normal tissues which normally undergo physiologic remodeling and angiogenesis: including bone, endometrium, ovary and mammary gland.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells. Persistent, unregulated angiogenesis also supports the pathological damage seen in these conditions. "Cancer" means angiogenesis-dependent cancers and tumors, *i.e.* tumors that require for their growth (expansion in volume and/or mass) an increase in the number and density of the blood vessels supplying then with blood. "Regression" refers to the reduction of tumor mass and size.

Angiogenesis-related diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemia, and tumor metastases; benign tumors, *e.g*, hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, *e.g.*, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophiliac joints; and angiofibroma. HMGCoA reductase inhibitors are also useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions

inhibitors are also useful in the treatment of diseases that have angiogenesis as a pathologic

consequence such as cat scratch disease (*Rochele minalia quintosa*) and ulcers (*Helobacter pylori*). A further discussion of angiogenesis-related diseases follows:

(a) Ischemia is associated with neovascularization and the release of VEGF.

(b) Blindness is one of the most devastating complications of diabetes. In one form of diabetic retinopathy, new vessel formation and proliferation of glial cells has been demonstrated as part of the retinal lesion. The aqueous humor of the eyes of animals made hypoxic by photocoagulation contains increased levels of VEGF (Miller *et al.*, 145 Am. J. Pathol. 574-84 (1994). Furthermore, in a preliminary study of diabetic patients treated with the HMGCoA reductase inhibitor pravastatin, significant improvement was found in the fundiscopic examination of all 6 patients studied compared to the untreated group (Gordon *et al.*, 112 Am. J. Ophthalmol. 385-91 (1991)).

(c) Rheumatoid arthritis is characterized by synovial membrane proliferation and outgrowth associated with erosion of articular cartilage and subchonral bone. The proliferating synovial membrane, the pannus, is vascularized by arterioles capillaries and venules. In collagen induced arthritis, an animal model for rheumatoid arthritis, the angiogenesis inhibitor AGM-1470 reversed pannus formation and neovasclarization as compared to control animals (Peacock *et al.* 175 J. Exp. Med. 1135-8 (1992)). An increase in VEGF has also been indicated in association with the angiogenesis of rheumatoid arthritis (Nagashima *et al.*, 22 J. Rheumatol. 1624-30 (1995)). Furthermore, the pro-angiogenic cytokine TNFα has been implicated in the pathogenesis of rheumatoid arthritis. In clinical trials, treatment of patients with an antibody to TNFα deactivated the endothelial cells in the synovium, to reduce the expression of adhesion molecules and to decrease the levels of VEGF in association with a marked improvement of disease (Nagashima *et al.*, 22 J. Rheumatol. 1624-30 (1995)). Thus, VEGF stimulated angiogenesis affects the pathogenesis of rheumatoid arthritis.

(d) Psoriasis is a common inherited skin disease that is characterized by hyperproliferation of epidermal keratinocytes and excessive dermal angiogenesis. Medium conditioned by the growth of keratinocytes from patients with psoriasis induces a marked angiogenic response in the rabbit corneal pocket assay (*see*, EXAMPLE 2 below for a description of the assay). Furthermore, keratinocytes from patients with psoriasis expressed increased levels of the pro-angiogenic cytokine IL-8 and a decrease in the anti-angiogenic thrombospondin (Nickoloff *et al.*, 144 Am. J. Pathol. 820-8 (1994)).

(e) Angiogenesis has also been shown to affect atherogenesis. The ingrowth of blood vessels into atherosclerotic plaques may contribute to an increase in plaque size, an increase in infiltration by white blood cells, and the resultant destabilization and rupture of the plaque leading to acute myocardial infarction (Moulton *et al.*, 99 Circulation 1726-1732 (1999)).

(f) Angiogenesis might affect the development of varicose veins. Several inhibitors of angiogenesis have been shown modulate the extent of venular dilation in an *in vivo* model.

In summary, angiogenesis is important for the pathogenesis of a number of inflammatory and proliferative diseases. Agents which interference with angiogenesis might affect the treatment of these diseases.

Birth Control Method

HMGCoA reductase inhibitors can be used as a birth control agent, by preventing the uterine vascularization required for blastocyst implantation and for development of the placenta. Thus, the invention provides an effective birth control method when an amount of HMGCoA reductase inhibitor sufficient to prevent embryo implantation is administered to a female. In one aspect of the birth control method, HMGCoA reductase inhibitor sufficient to block embryo implantation is administered before or after intercourse and fertilization have occurred, thus providing an effective method of birth control, possible a "morning after" method. Inhibition of vascularization of the uterine endometrium interferes with implantation of the blastocyst. Similar inhibition of vascularization of the mucosa of the uterine tube interferes with implantation of the blastocyst, preventing occurrence of a tubal pregnancy. Administration methods of HMGCoA reductase inhibitors may include, but are not limited to, pills, injections (intravenous, subcutaneous, intramuscular), suppositories, vaginal sponges, vaginal tampons, and intrauterine devices. HMGCoA reductase inhibitor administration also interferes with normal enhanced vascularization of the placenta.

Formulation and Dosage

The HMGCoA reductase inhibitor of the invention can be provided in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intravenous, intraspinal, subcutaneous or intramuscular routes. In addition, the HMGCoA reductase inhibitor may be incorporated into biodegradable polymers allowing for sustained

release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the HMGCoA reductase inhibitor is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of HMGCoA reductase inhibitor through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor. The biodegradable polymers and their use are described, for example, by Brem *et al.*, 74 J. Neurosurg. 441-446 (1991).

HMGCoA reductase inhibitor formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

The HMGCoA reductase inhibitor formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carriers or excipients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Therapeutically and prophylactically effective dosages of HMGCoA reductase inhibitor can be determined by those of skill in the art. The dosage of the HMGCoA reductase inhibitor depends on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating humans, between approximately 0.5 mg/kg to 500 mg/kg of the HMGCoA reductase inhibitor can be administered. The preferred range for HMGCoA reductase inhibitor administration for reducing serum cholesterol is oral administration of from 10-40 mg/day. Pravastatin is typically administered orally at a dose of 40 mg/day (West of Scotland Coronary Prevention Study Group, 97 Circulation 1440-5 (1998); Sacks *et al.*, 97

Circulation 1446-52 (1998)) for reducing hypercholesterolemia. The recommended starting dose is 10 or 20 mg once daily at bedtime.

Therapeutic doses of simvastatin result in serum levels of 0.02-0.27 μM (Desager & Horsmans, 31 Clin. Pharmacokinet 348 (1996)). In the EXAMPLES provided below, these concentrations had significant effects on cell division, cell migration and the formation of capillary-like structures by HUVECs. (*see,* EXAMPLE 1). Furthermore, the effects of HMGCoA reductase inhibitors are time dependent and hence lower doses given to patients over months and years are likely to have similar anti-angiogenic effects. In the corneal pocket and CAM assays, simvastatin suppressed bFGF and VEGF stimulated angiogenesis at somewhat higher concentrations than those seen *in vitro*. This may be because in these models, delivery of simvastatin is via diffusion from the pellet or mesh, which limits the effective concentration of the drug. However, this result could have clinical significance for humans, indicationg that the therapeutic or prophylactic dosage levels for the methods of this invention are higher than for the the levels for previous, cholesterol reducing, usages of statins.

In general, a "standard therapeutic dosage" can be 5 to 40 mg/day of a statin, such as is described in the paragraphs and citations provided above. In general, a "higher than standard terapeutic dosagecan be a dose of as high as 120 mg/day or higher statin, such as is described in the paragraphs and citations provided above. In general, a "lower than standard terapeutic dosage" is a concentration as low as 0.5 M, as is shown in the EXAMPLES below.

Guidance for therapeutically and prophylactically effective dosages of HMGCoA reductase inhibitors for anti-angiogenesis can differ from the dosage recommended for reducing hypercholesterolemia. Guidance for therapeutically and prophylactically effective dosages of HMGCoA reductase inhibitors can be determined by *in vivo* and *in vitro* assays. For example, HMGCoA reductase inhibitors may be quickly and easily tested *in vitro* for endothelial proliferation-inhibiting activity using a biological activity assay such as the bovine capillary endothelial cell proliferation assay (*see,* United States patents 5,885,795 and 5,854,205, both to O'Reilly *et al.*, both incorporated herein by reference). Other *in vitro* bioassays include the chick chorioallantoic membrane (CAM) assay and the mouse corneal assay. The chick chorioallantoic membrane assay is described by O'Reilly *et al.*, 79(2) Cell

In vivo assays include the effect of administering anti-angiogenic factors on implanted tumors. Assays can be performed to test to what extent an HMGCoA reductase inhibitor reduces microvessel density and causes inhibition of human tumor growth in nude mice, such as was performed by Kim et al., 362 Nature 841-844 (1993). Assays can also be performed to test to what extent an HMGCoA reductase inhibitor causes inhibition of growth of a mouse tumor such as was performed by Hori *et al.*, 51 Cancer Research 6180-6184 (1991).

The *in vivo* effect of HMGCoA reductase inhibitors can be tested in genetically engineered mouse models of cancer. One strength of these models is that cancers arise from normal cells in their natural tissue microenvironments and progress through multiple stages, as does human cancer. Such models of organ-specific cancer also present opportunities for development not only of cancer therapies but also of preventative strategies that block the progression of premalignant lesions into tumors. The RIP1-Tag2 transgenic mouse model of pancreatic islet carcinogenesis serves as a general prototype of the pathways, parameters, and molecular mechanisms of multistage tumorigenesis and of methods for treating tumors with anti-angiogenic factors (*see,* Bergers *et al.*, 284(5415) Science 808-812 (1999)).

Guidance for determining the therapeutically and prophylactically effective dosages of HMGCoA reductase inhibitor is also provided in EXAMPLES 6-9. The goals of EXAMPLES 6-9 are to determine the molecular interactions by which lipid metabolism and angiotensin II regulate angiogenesis and contribute to the development of atherosclerosis. In these EXAMPLES, we test how signaling by VEGF and the potentiation of VEGF signaling by integrins and angiotensin II are each dependent on a member of the Rho family of GTPases and that HMGCoA reductase inhibitors interfere with angiogenesis by inhibiting the posttranslational lipidation of Rho GTPases. We further test how HMGCoA reductase inhibitors interfere with the VEGF signaling pathway and angiogenesis in an *in vivo* model of atherosclerosis and decrease the neo-vascularization and size of atherosclerotic plaques. These EXAMPLE provide guidance for a new relationship between lipid metabolism, growth factor signaling and hypertension, which could have important implications for the treatment of atherosclerosis. Specifically we provide guidance for testing the therapeutically effective or prophylactically effective dosage by assessing four major points:

(1) That VEGF, bFGF, and extracellular matrix-stimulation of angiogenesis are dependent on the geranylgeranylation of a Rho GTPase. Specifically, we provide a methodical plan for assessing therapeutic dosage by showing (a) that stimulation of angiogenesis by

VEGF in the chick chorioallantoic membrane (CAM) and by bFGF in the mouse cornea is dependent on the posttranslational lipidation of a Rho GTPases; and (b) that the cellular response to VEGF, specifically VEGF stimulation of endothelial cell invasion, migration, and tube formation are dependent on the posttranslational lipidation of a Rho GTPase and inhibited by HMGCoA reductase inhibitors;

(2) That VEGF signaling is dependent on a Rho GTPase and inhibited by HMGCoA reductase inhibitors at two levels: at the level of receptor activation and at the level of gene expression. Specifically, we provide a methodical plan for assessing therapeutic dosage by assaying for (a) that VEGF-stimulation of tyrosine phosphorylation of VEGF receptors, Flt-1, Flk-1/KDR, is regulated by a member of the Rho family of GTPases; and (b) that induction of VEGF, Flt-1 and Flk-1/KDR expression by angiotensin II, thrombin and hypoxia requires the Rho-dependent activation of a MAP kinase pathway. Hence, VEGF receptor activation and expression of VEGF and VEGF receptors are regulated by the posttranslational lipidation of Rho GTPases and inhibited by HMGCoA reductase inhibitors;

(3) That activation of integrin signaling potentiates VEGF signaling via Rho-dependent pathways and HMGCoA reductase inhibitors disrupt the cross-talk between VEGF and integrin signaling. Specifically we provide a methodical plan for assessing therapeutic dosage by showing (a) that VEGF stimulation of FAK phosphorylation is dependent on a Rho GTPase; (b) that the effects of VEGF on endothelial cell invasion and migration are dependent in part on FAK; (c) that integrin-potentiation of VEGF stimulated phosphorylation of VEGF receptors is dependent on Rho and mediated through FAK; and (d) that integrin-stimulation of VEGF expression is dependent on the activation of a Rho dependent MAP kinase pathway.

(4) That HMGCoA reductase inhibitors decrease the growth and size of atherosclerotic plaques by inhibiting the expression of VEGF and VEGF receptors and interfering with angiogenesis in an animal model of atherosclerosis. Using cholesterol-fed Apo-E-/- mice, we provide guidance for showing (a) that HMGCoA reductase inhibitors interfere with the expression of VEGF, Flt-1 and Flk-1/KDR in parallel with a decreased in neo-vascularization and plaque size; and (b) that angiotensin II treatment induces the expression of VEGF, Flt-1

Other embodiments of the invention.

The invention provides a method for identifying an inhibitor of angiogenesis. The practice of the method can be further detrmined using the guidance provided in the EXAMPLES below. The steps of the method include: (a) assaying the cellular response of endothelial cells to an angiogenic factor; (b) assaying the cellular response of endothelial cells to an angiogenic factor in the presence of an HMGCoA reductase inhibitor, such that the presence of the HMGCoA reductase inhibitor inhibits the cellular response of the endothelial cells; (c) assaying the cellular response of endothelial cells to an angiogenic factor in the presence of a test compound; and (d) comparing the cellular response of endothelial cells from step (a) with the cellular response of endothelial cells from step (b) and the cellular response of endothelial cells from step (c). An inhibition of the cellular response of endothelial cells from step (c) as compared with the cellular response of endothelial cells from step (a) identifies the test compound as an inhibitor of angiogenesis.

The invention provides another method for identifying an inhibitor of angiogenesis. The practice of this method can also be further detrmined using the guidance provided in the EXAMPLES below. The steps of the method include: (a) assaying the activity of small GTP-binding protein activity from an endothelial cell; (b) assaying the activity of small GTP-binding protein activity from an endothelial cell that has been contacted with an HMGCoA reductase inhibitor, wherein the contact by the HMGCoA reductase inhibitor inhibits the activity of small GTP-binding protein activity in the endothelial cell; (c) assaying the activity of small GTP-binding protein activity from an endothelial cell that has been contacted with a test compound; and (d) comparing the activity of small GTP-binding protein activity from an endothelial cell from step (a) with the activity of small GTP-binding protein activity from an endothelial cell from step (b) and the activity of small GTP-binding protein activity from an endothelial cell from step (c). An inhibition of the activity of small GTP-binding protein activity from an endothelial cell from step (c) as compared with the activity of small GTP-binding protein activity from an endothelial cell from step (a) identifies the test compound as an inhibitor of angiogenesis.

The invention provides yet another method for identifying an inhibitor of angiogenesis. The practice of this method can be further detrmined using the guidance provided in the EXAMPLES below. The steps of the method include: (a) assaying the formation of organized structures *in vitro* by endothelial cells; (b) assaying the formation of organized structures *in vitro* by endothelial cells in the presence of an HMGCoA reductase inhibitor, wherein the

presence of the HMGCoA reductase inhibitor inhibits the formation of organized structures *in vitro* by endothelial cells; (c) assaying the formation of organized structures *in vitro* by endothelial cells in the presence of a test compound; and (d) comparing the formation of organized structures *in vitro* by endothelial cells from step (a) with the formation of organized structures *in vitro* by endothelial cells from step (b) and the formation of organized structures *in vitro* by endothelial cells from step (c) An inhibition of the formation of organized structures *in vitro* by endothelial cells from step (c) as compared with the formation of organized structures *in vitro* by endothelial cells from step (a) identifies the test compound as an inhibitor of angiogenesis.

The invention provides yet another method for identifying an inhibitor of angiogenesis. The practice of this method can be further detrmined using the guidance provided in the EXAMPLES below. The steps of the method include: (a) assaying the formation of blood vessels *in vivo*; (b) assaying the formation of blood vessels *in vivo* in the presence of an HMGCoA reductase inhibitor, wherein the presence of an HMGCoA reductase inhibitor inhibits the formation of blood vessels; (c) assaying the formation of blood vessels *in vivo* in the presence of a test compound; and (d) comparing the formation of blood vessels in step (a) with the formation of blood vessels in step (b) and the formation of blood vessels in step (c). An inhibition of the formation of blood vessels in step (c) as compared with the formation of blood vessels in step (a) identifies the test compound as an inhibitor of angiogenesis.

The invention provides an article of manufacture (a kit), comprising packaging material and a primary reagent contained within said packaging material. The primary reagent is an HMGCoA reductase inhibitor, as described above. The packaging material includes a label which indicates that the primary reagent can be used for reducing angiogenesis in the tissue of a host (such as is also descibed above).

The details of one or more embodiments of the invention are set forth in the accompanying description above. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials have been described. Other features, objects, and advantages

[illegible] the appended claims. As used in this specification and the appended claims, the singular forms include plural referents unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein

have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All patents and publications cited in this specification are incorporated by reference.

The following EXAMPLES are presented to more fully illustrate the preferred embodiments of the invention. These EXAMPLES should in no way be construed as limiting the scope of the invention, as defined by the appended claims.

EXAMPLE 1
THE ANTI-ANGIOGENIC EFFECTS OF HMGCoA REDUCTASE INHIBITORS *IN VITRO* SHOWS A NEW ROLE FOR GERANYLGERANYLATED PROTEINS IN THE REGULATION OF ANGIOGENESIS

HMGCoA reductase inhibitors inhibited angiogenesis in vitro. This EXAMPLE demonstrates that HMGCoA reductase inhibitors interfere with the proliferation and migration of HUVECs in culture and their differentiation into blood vessel-like structures. Endothelial cells have a critical role in the development of new blood vessels. In response to vascular endothelial growth factor (VEGF) endothelial cells divide, migrate and differentiate into elongated tubular structures which become blood vessels. To determine the effect of regulation of the cholesterol metabolic pathway on angiogenesis, we tested the effects of HMGCoA reductase inhibitors, simvastatin and atorvastatin on the formation of capillary-like structures by human umbilical vein endothelial cells (HUVECs) cultured on Matrigel. Within 16 hr of plating, cells differentiated into a series of capillary-like structures (FIG. 1A). Matrigel® (Collaborative Research) is a basement membrane extract enriched with laminin. Matrigel® has the ability to promote the differentiation of endothelial cells into capillary like structures. When HUVECs are incubated for several hours on plates precoated with the extracellular matrix extract Matrigel®, they arrange themselves into polygonal structures with walls composed of single HUVECs. In the presence of low concentrations of simvastatin (0.1 μM, 16 hr incubation) added at the time of plating, the walls of these capillary-like structures became thickened and multicellular (FIG. 1B). At higher concentrations, simvastatin disrupted the organization of the capillary-like structures in a dose-dependent manner. (FIG. 1C, 1 μM; FIG. 1D, 5 μM). Atorvastatin had a similar effect. Thus, the inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors interfered with angiogenesis in HUVECs *in vitro*.

HUVECs were isolated using the method of Gimbrone, 3 Prog Hemost. Thromb. 1 (1976) and cultured in medium M199 supplemented with 20% FBS, 2 mM L-glutamine, 50 μg/ml endothelial cell growth factor, 100 μg/ml heparin and 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were used after the third passage. For tests of cell proliferation, HUVECs were plated at $1x10^5$ cells per 60 mm dish with various concentrations of simvastatin. After incubation for three days at 37° C in 5% CO_2, cells were trypsinized and viable cells determined by Trypan Blue exclusion. For growth of cells on Matrigel 6-well plates were coated with Matrigel (Collaborative Research, Inc., MA, USA), an extract of basement membrane secreted by the Englebreth-Holm-Swarm murine sarcoma containing a high concentration of laminin, and allowed to gel for one hour at 37° C. HUVECs, $5x10^5$, were added to each well with various concentrations of simvastatin and incubated for 16-24 hr. The effect on the formation of capillary-like structures was determined by phase contrast microscopy.

Effect of simvastatin on the proliferation and migration of endothelial cells. Angiogenesis involves proliferation, migration and differentiation of endothelial cells (Folkman & Klagsbrun, 235 Science 442-7 (1987)). To determine the effect of simvastatin on the proliferation of HUVECs, cells ($1x10^5$ cells/60 mm dish) were plated and incubated at various concentrations of simvastatin. After 3 days, cells were trypsinized and counted. Simvastatin decreased cell number in a dose-dependent manner with a 33% decrease at 0.1 μM and complete inhibition of cell growth at 2 μM (FIG. 2A).

The effect of simvastatin on migration of HUVECs cells was also tested using a cell-motility assay (FIG. 2B). For the migration tests, HUVECs cultured on 60 mm dishes were pretreated with various concentrations of simvastatin for 16 hours followed by a 1 hr incubation with 5 μM Calcein-AM (Molecular Probes). Cells were washed, trypsinized, and resuspended in M199 medium. The labeled cells were added to 3.0 μm FluoroBlock inserts (FALCON) at a density of 50,000 cells/insert in the presence of the indicated concentrations of simvastatin. Medium M119 supplemented with 10% FBS was used as a chemo-attractant in the lower wells, while medium M119 alone was added to the control wells. Inserts were incubated for 2 hours at 37°C and fl[illegible]

[illegible] wavelengths of 485 [illegible] nm. Medium M199 alone is added to the upper chamber, while 1% serum with and without VEGF is added to the lower chamber. Migration is determined by

measuring the fluorescence of cells which migrate through the UV blocked membrane using a cytoFluor 4000 plate reader at excitation/emission wave lengths of 485/530 nm.

Using a fluorescence assay, incubation of cells with simvastatin also demonstrated inhibition of cell migration in a dose-dependent manner, 54±3% (±SEM, N=3) at 5μM simvastatin, which was significant at 0.5 μM, $p<0.01$. The more hydrophilic HMGCoA reductase inhibitor pravastatin, whose accessibility to non-liver cells is limited (Arai *et al.*, 40 Sankyo Kenkyusho Nenpo 1-38), had no effect on the formation of capillary-like structures at concentrations as high as 20 μM.

Inhibition of migration was significant at 0.5 μM, $p<0.01$.

The finding that HMGCoA reductase inhibitors, simvastatin and atorvastatin, but not pravastatin inhibited angiogenesis *in vitro* might reflect that fact that although all three of these HMGCoA reductase inhibitors are quite similar in structure they exhibit markedly different hydrophobicities: simvastatin>atorvastatin>pravastatin. Although all three are transported into the liver, uptake into non-liver cells is dependent on relative hydrophobicity, which may account for differences in anti-angiogenic effect *in vitro*. The finding that pravastatin appeared to decrease the number of blood vessels in atherosclerotic lesions of cholesterol-fed monkeys *in vivo*, indicates that *in vivo* pravastatin or a metabolite of pravastatin may effect endothelial cell function.

EXAMPLE 2
EFFECT OF HMGCoA REDUCTASE INHIBITORS ON VEGF AND FGF-2-MEDIATED ANGIOGENESIS

HMGCoA reductase inhibitors interfered with angiogenesis in vivo. To directly test the effects of simvastatin on angiogenesis, two models were used. The effects of simvastatin on VEGF stimulated angiogenesis were tested in a chorioallontic membrane (CAM) model of Nguyen *et al.*, 47 Microvasc. Res. 31-40 (1994) and FGF-2-stimulated angiogenesis in a corneal pocket model. This EXAMPLE shows that HMGCoA reductase inhibitors interfere with VEGF and FGF-2 stimulation of blood vessel formation in both models. In a chick chorioallantoic membrane (CAM) assay, the angiogenic response to VEGF was determined by computer-assisted imaging of the number of blood vessels that grew into a matrix polymer containing the angiogenic factor. Four independent determinations indicated that the HMGCoA reductase inhibitor simvastatin suppressed angiogenesis induced by VEGF in a dose-dependent manner.

Chorioallontic Membrane Model. CAM assay was performed as described by Vazquez *et al.*, 274 J. Biol. Chem. 23349 (1999). Leghorn chicken embryos (Spafas) 12-14 days *in ovo* were used. Matrigel (750 μm/ml), VEGF, 250 ng/mesh alone or mixed with the indicated concentrations of simvastatin were loaded onto nylon mesh (pore size 250 μm; Tetko Inc.) incubated at 37°C for 30 min and 4°C for 2 hr to allow polymerization. For example, VEGF and other agents can be suspended at the desired concentrations in a mixture of aluminum sucrose octasulfate (sucralfate) which had been previously sterilized in boiling double-distilled water and Vitrogen (type I collagen) which had been diluted with water and neutralized with 0.1 M NaOH. A 20 μl aliquot of this suspension is deposited onto a piece of mesh cut to the desired dimensions. The sample is allowed to gel on the top of the flat end of a 1/8-inch-diameter Teflon rod cut into 1.2 cm length rods and mounted on a 100 mm petri dish. The dish is incubated at 37° C at 65-70% humidity for 20 min.

Meshes were placed on the CAM and incubated for 24 hr. For example, the sample can be then transferred onto the CAM of a 8-day chick embryo. A smaller piece of mesh is placed on top of the collagen gel and incubation continued.

Vessels were visualized by injecting 400 μl of fluorescein isothiocynate dextran into the embryo. Chicks were fixed with 3.75% formaldehyde and meshes dissected and mounted on slides. For example, the mesh is observed from day 3 to day 9 after implantation with a Zeiss stereoscope microscope. The stimulation of angiogenesis is expressed as a percentage of the squares in the top mesh which contains blood cells. The fluorescence intensity is analyzed with a computer-assisted image program (NIH Image 1.59, (Vazquez *et al.*, 274 J. Biol. Chem. 23349-57 (1999)).

Quantitation of the capillary growth demonstrated a 2.7-fold increase in angiogenic response in the presence of 250 ng VEGF as compared to control. Treatment with VEGF plus increasing concentrations of simvastatin demonstrated a dose-dependent decrease in the angiogenic response. Effects were seen at concentrations of simvastatin as low at 0.5μM (*see*, FIG. 3). The response at 10 μM simvastatin was not statistically significantly different from control levels in chorioallantoic membranes treated with vehicle only (FIG. 3).

Mouse Corneal Pocket Assay. The corneal pocket assay also demonstrated that

[illegible] angiogenesis in the corneal micropocket assay. The corneas of mice were implanted with a polymer containing 10 ng of FGF-2 with and without either 5 μM or 10 μM

simvastatin. In the absence of simvastatin, this concentration of FGF-2 induced the formation of numerous capillaries (FIG. 4A).

For the corneal pocket assay, Swiss Webster mice (Charles River Boston) were used at 8 to 10 weeks of age for implantation of Hydron pellets. Cornea pockets were performed as described by Kenyon *et al.*, 37 Invest. Ophthalmol. Vis. Sci. 1625 (1996), Loughman *et al.*, 24 Aust. N. Z. J. Ophthalmol. 289-95 (1996). Pellets were generated by mixing 10 μg of recombinant FGF-2 plus 1 mg of sucralfate and 10 μl of Hydron (200 mg/ml in ethanol: New Brunswick, New Jersey) and the indicated concentration of simvastatin (or C3 exotoxin or geranylgeranylpyrophosphate, or farnesylpyrophosphate; *see*, below, EXAMPLES 7-10). The suspension was smeared onto a sterile nylon mesh square (pore size 500 μm; Tetko Inc.) and allowed to dry for 30 min. The fibers of the mesh were pulled were pulled to produce pellets of 500 μm^3 that were stored at -20°C. Five days after implantation corneal angiogenesis was photographed and the presence of vessels determined.

This angiogenesis was almost totally reversed in corneas in which pockets were treated with beads impregnated with both simvastatin and FGF-2 (FIG. 4B). The presence of 5 μM simvastatin completely reversed the effects of FGF-2 (FIG. 4B). The letter "P" in FIG. 4 is shown to indicate the position of the polymer. Of 29 corneas treated with FGF-2, 28 demonstrated an angiogenic response to FGF-2. Of the 29 corneas treated with FGF-2 plus 5 μM simvastatin, the angiogenic response was blocked in 26 corneas.

To better visualize the effects of simvastatin on corneal vascularity, corneas were treated as in FIG. 4A. The mouse's tail was also injected with tomato lectin to visualize the blood vessels. Sagittal sections of the mouse eye were fixed and the vascular bed visualized by photomicrography. FIG. 4C (top panel) demonstrates the vascular bed in a controlled cornea. FIG. 4D demonstrates the effects of 10 ng of FGF-2 following 48 hr after the insertion of the polymer. The effect of the pellet alone is shown in FIG. 4D. Addition of 100 ng FGF-2 into the corneal pocket resulted in the marked proliferation of small capillaries. This effect of FGF-2 was significantly suppressed by 5 μM simvastatin and completely inhibited by 10 μM simvastatin. The effect of 10 ng of FGF-2 plus simvastatin (5 μM and 10 μM) on vascularity is shown in FIG. 4E and FIG. 4F. Simvastatin decreased capillary growth back to control levels in a dose-dependent manner.

EXAMPLE 3
FURTHER EFFECTS OF HMGCoA REDUCTASE INHIBITORS ON VEGF AND FGF-2-MEDIATED ANGIOGENESIS

The HMGCoA reductase inhibitor simvastatin interfered with VEGF signaling. Assays were carried out to determine whether simvastatin interfered with VEGF signaling via an effect on the ligand-induced autophosphorylation of Flt-1 and Flk-1/KDR. In HUVECs incubated in 1% serum without added growth factors, a 5 min incubation with 10 ng/ml VEGF resulted in a marked increase in tyrosine phosphorylation of Flk-1/KDR, measured by immunoprecipitation with antibody to the receptor followed by Western blot analysis with an anti-phosphotyrosine antibody. A 16 hr incubation of cells with increasing concentrations of simvastatin resulted in a marked dose dependent decrease in the tyrosine phosphorylation of Flk-1/KDR, while simvastatin had no effect on the expression of the receptor, as measured by Western blotting of aliquots of the same cell extracts with an antibody to Flk-1/KDR. These data are typical of 4 similar assays. In a similar assays, we demonstrated that simvastatin significantly decreased the VEGF-stimulated tyrosine phosphorylation of Flt-1 while having no effect on total Flt-1 protein. These data are typical of three similar assays.

These results indicated that the inhibition of the cholesterol metabolic pathway by the HMGCoA reductase inhibitor simvastatin interfered with the VEGF activation of Flt-1 and Flk-1/KDR, but not the expression of these receptors. To determine whether HMGCoA reductase inhibitors interfered with cross-talk between VEGF and integrin signaling, we determined the effect of simvastatin on VEGF-stimulated tyrosine phosphorylation of focal adhesion kinase (FAK). Cells were incubated overnight in 2% serum with and without simvastatin followed by a 5 min incubation with VEGF. Immunoprecipitation with anti-FAK antibody followed by Western blotting with anti-phosphotyrosine antibody demonstrated that VEGF-stimulated tyrosine phosphorylation of FAK and simvastatin decreased both basal and VEGF-stimulated phosphorylation of FAK, whereas simvastatin had no effect on the expression of FAK.

Thus, HMGCoA reductase inhibitors might interfere with the cross-talk between VEGF and integrin signaling. In this EXAMPLE, we test how VEGF stimulation of FAK

reductase inhibitor interfered [illegible] signaling [illegible] the cross-talk between VEGF and integrin signaling.

HMGCoA reductase inhibitors interfere with angiotensin II-stimulation of VEGF expression. The expression of VEGF is known to be regulated by growth factors, cytokines, hypoxia and the activation of integrins. To determine whether inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors interferes with VEGF signaling and angiogenesis by regulating the expression of VEGF, we tested the effect of simvastatin on the expression of VEGF in HUVECs. HUVECs were incubated with or without simvastatin (1 μM, 16 hr) followed by a 5 hr incubation with thrombin (10 U/ml) or angiotensin II (100 nM). We here demonstrate that simvastatin significantly decreased the expression of both thrombin and angiotensin II induced VEGF expression.

To further characterize the geranylgeranylated protein involved in angiogenesis, we incubated cells with C3 exo-toxin, which inhibits the activity of Rho GTPases by catalyzing the ADP-ribosylation of Rho family members and is relatively specific for Rho A, B and C (Aktories, 5 Trends Microbiol. 282 (1997)). Treatment of HUVECs with C3 exo-toxin at the time of plating on Matrigel mimicked the effect of simvastatin and disrupted the formation of capillary-like structures (FIG. 5). C3 exotoxin mimicked the effect of simvastatin on angiotensin II-induced expression of VEGF. These data are typical of two other studies.

This EXAMPLE shows that the formation of capillary-like structures depends on a protein of the Rho family of small GTP binding proteins and that simvastatin interferes with this dependence by inhibiting the geranylgeranylation of Rho.

EXAMPLE 4
REVERSAL OF THE EFFECT OF SIMVASTATIN ON THE FORMATION OF VASCULAR-LIKE STRUCTURES BY HUVECs IN THE PRESENCE OF GERANYLGERANYLPYROPHOSPHATE

This EXAMPLE shows that the effects of HMGCoA reductase inhibitors on angiogenesis in endothelial cells is mediated through the actions of geranylgeranylated proteins, such as the family of small GTP binding proteins. In this EXAMPLE, HMGCoA reductase inhibitors exert their anti-angiogenic effects by the interference with the lipidation of small GTP-binding proteins such as Rho.

Since geranylgeranylpyrophosphate and farnesylpyrophosphate, which are substrates for the enzymes which catalyze to farnesylation and geranylgeranylation of proteins, should be able to reverse the effects of simvastatin on protein lipidation, we tested the effects of these compounds on the interference of simvastatin with the development of capillary-like structures

in HUVECs grown on Matrigel®. Comparison of cells incubated with 5 μM simvastatin plus 10 μM farnesylpyrophosphate or 10 μM geranylgeranylpyrophosphate (GGPP) with cells incubated with simvastatin alone demonstrated that farnesylpyrophosphate had no effect on simvastatin interference with capillary structure formation, while geranylgeranylpyrophosphate completely reversed the effects of simvastatin on capillary structure-formation, cells treated with simvastatin plus geranylgeranylpyrophosphate and cells treated with simvastatin alone.

The results of this EXAMPLE strongly support the conclusion that HMGCoA reductase inhibitors, at pharmacologically relevant concentrations exert a significant anti-angiogenic effect. This EXAMPLE provides additional guidance as to the level of HMGCoA reductase inhibitor in a therapeutic dose.

The finding in this EXAMPLE that geranylgeranylpyrophosphate reversed the effects of simvastatin on the formation of capillary-like structures by HUVECs supports the conclusion that a geranylgeranylated protein has an important role in the angiogenic response of HUVECs platted on Matrigel®. Thus, inhibition of the geranylgeranylation reaction by HMGCoA reductase inhibitors is responsible for the interference of simvastatin with the formation of capillary-like structures. Taken together with the findings in EXAMPLE 2 that simvastatin interferes with angiogenesis as measured by the chorioallantoic membrane and corneal pocket assays in an *in vivo* model, these data show that simvastatin interferes with angiogenesis by a cholesterol-independent effect.

The results of this EXAMPLE have important implications for the treatment of patients with diseases whose pathogenesis is dependent on neovascularization. The lack of significant side-effects of HMGCoA reductase inhibitors combined with their efficacy in the reduction of coronary events have made these agents important tools in the treatment and prevention of coronary artery disease. The addition of these newly described anti-angiogenic properties provide exciting new possibilities for their therapeutic use in the treatment and prevention not only of atherosclerosis, but also of cancer, arthritis and diabetic retinopathy.

EXAMPLE 5
FURTHER REVERSAL OF THE EFFECT OF SIMVASTATIN ON THE FORMATION OF VASCULAR-LIKE STRUCTURES BY HUVECs IN THE PRESENCE OF GERANYLGERANYLPYROPHOSPHATE

The anti-angiogenic effect of the HMGCoA reductase inhibitor simvastatin in vitro involved the inhibition of the geranylgeranylation of Rho. The inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors limits the availability of farnesylpyrophosphate which is a common precursor to 5 different pathways: synthesis of cholesterol, dolichol, ubiquinone, and pathways for posttranslational lipidation of proteins by farnesylpyrophosphate and geranylgeranylpyrophosphate. To determine which branch of the cholesterol metabolic pathway was responsible for simvastatin inhibition of capillary-like structure formation in HUVECs cultured on Matrigel, we tested the effects of specific inhibitors of three of these pathways.

To determine which branch of the cholesterol metabolic pathway was responsible for the effect of simvastatin on the formation of capillary-like structures in HUVECs cultured on Matrigel, we tested the effects of specific inhibitors of three pathways downstream from farnesylpyrophosphate. TMD, an inhibitor of the conversion of squalene to lanosterol, which interfered with cholesterol biosynthesis (Chang *et al.*, 254 J. Biol. Chem. 11258 (1979); Lerner *et al.*, 270 J. Biol. Chem. 26802 (1995); Vogt *et al.*, 272 J. Biol. Chem. 27224 (1997)), had no effect on the formation of capillary-like structures in HUVECs cultured on Matrigel. Incubating cells overnight on Matrigel with FTI-277, a specific inhibitor of protein farnesyltransferase, the enzyme which catalyzes the covalent binding of farnesylpyrophoshate to small GTP binding proteins such as Ras (Casey & Seabra, 271 J. Biol. Chem. 5289-92 (1996); Chang *et al.*, 254 J. Biol. Chem. 11258 (1979); Lerner *et al.*, 270 J. Biol. Chem. 26802 (1995); Vogt *et al.*, 272 J. Biol. Chem. 27224 (1997)), also had no effect on the formation of capillary-like structures. However, GGTI-288, a specific inhibitor of geranylgeranyltransferase, the enzyme which catalyzes the geranylgeranylation of small GTP binding proteins such as Rho (Chang *et al.*, 254 J. Biol. Chem. 11258 (1979); Lerner *et al.*, 270 J. Biol. Chem. 26802 (1995); Vogt *et al.*, 272 J. Biol. Chem. 27224 (1997)), mimicked the effect of simvastatin on the formation of capillary like structures.

Inhibition of the farnesylation and geranylgeranylation of proteins by HMGCoA reductase inhibitors is reversed by incubation of cells with farnesylpyrophosphate (FPP), the substrate for farnesyltransferase, and geranylgeranylpyrophosphate (GGPP), the substrate for

geranylgeranyltransferase, respectively. Farnesylpyrophosphate had no effect on disruption of the formation of capillary-like structures by the HMGCoA reductase inhibitor simvastatin, whereas geranylgeranylpyrophosphate completely reversed the effects of simvastatin. Finally, we tested the effect *of C. botulinum* C3 exotoxin, which specifically ADP ribosylates and inactivates the Rho family of small GTPases (Aktories, 5 Trends Microbiol. 282-8 (1997)). Treatment of HUVECs with C3 exotoxin at the time of plating on Matrigel mimicked the effect of simvastatin and disrupted the formation of capillary-like structures (*see*, FIG. 5). These data support the conclusion that simvastatin interfered with the formation of capillary-like structures by HUVECs grown on Matrigel by inhibiting the posttranslational geranylgeranylation of the Rho family of small GTP binding proteins. In this EXAMPLE, we test this using recombinant adenoviruses expressing dominant active and dominant negative mutants of the Rho family of GTPases, that the formation of capillary-like structures by HUVECs is dependent at least in part on a members of the Rho family of GTP binding proteins.

The finding that the anti-angiogenic effects of simvastatin are reversed by GGPP, the substrate for geranylgeranyltransferase, and mimicked by GGTI-288, a specific inhibitor of geranylgeranyltransferase, show that HMGCoA reductase inhibitors interfere with angiogenesis via the inhibition of the geranylgeranylation reaction.

The finding that C3 exotoxin which interferes with the function of Rho also inhibits the formation of capillary-like structures further shows the effect of a Rho GTPase in angiogenesis. These data are in agreement with a study in transformed endothelial cells from rat liver sinusoids, in which small GTP binding proteins were involved in the formation of tubular-like structures (Maru *et al.*, 176 J. Cell. Physiol. 223 (1998)). Although Rho has been implicated in processes such as cell division and cell migration which affect angiogenesis (Aepfelbacher *et al.*, 17 Arterioscler. Thromb. Vasc. Biol. 1623 (1997)), the direct involvement of Rho in angiogenesis has not previously been demonstrated.

EXAMPLE 6
VEGF-STIMULATION, bFGF-STIMULATION AND EXTRACELLULAR

In this EXAMPLE, we test how VEGF-stimulated angiogenesis in the CAM and bFGF stimulated angiogenesis in the mouse corneal pocket are dependent on specific members of the

Rho family of GTPases. Furthermore, we test how the cellular response of endothelial cells to VEGF. We test how cell invasion, cell migration, and tube formation is dependent on the geranylgeranylation of Rho. This EXAMPLE provides guidance for testing how HMGCoA reductase inhibitors exert an anti-angiogenic via the inhibition of the geranylgeranylation of a member of the Rho family of GTPases. Thus, this EXAMPLE provides guidance for testing how to determine therapeutic or prophylactic dosages of HMGCoA reductase inhibitors.

HMGCoA reductase inhibitors inhibit angiogenesis via an effect on the geranylgeranylation of a Rho GTPase. We use GGTI, a specific inhibitor of geranylgeranyltransferase; FTI, a specific inhibitor of farnesyl protein transferase; geranylgeranylpyrophosphate, the substrate for gernylgernyltransferase or farnesylpyrophosphate, the substrate for farnesyltransferase and C3 exotoxin which ADP-ribosylates Rho and interferes with its function. If (as expected) inhibition of the cholesterol metabolic pathway by simvastatin interferes with VEGF-stimulated or bFGF-stimulated angiogenesis by inhibiting the geranylgeranylation of Rho, then GGTI and C3 exotoxin mimics the effect of simvastatin on angiogenesis and geranylgeranylpyrophosphate reverses the effects of simvastatin on angiogenesis.

Dependence of bFGF-stimulated angiogenesis in the mouse corneal pocket assay on protein geranylgeranylation. We compare the angiogenic response to bFGF in the mouse corneal pocket assay using pellets containing bFGF alone; bFGF plus 5 μM simvastatin; bFGF plus 10 μM GGTI bFGF plus 5 μM simvastatin plus 10 μM geranylgeranylpyrophosphate or farnesylpyrophosphate. If (as expected) bFGF stimulated angiogenesis is dependent on protein geranylgeranylation, then GGTI should mimic the effects of simvastatin. If (as expected) simvastatin suppression of bFGF stimulated angiogenesis is due to inhibition of protein geranylgeranylation, then geranylgeranylpyrophosphate restores the bFGF angiogenic response. Should there be no response to geranylgeranylpyrophosphate at 10 μM, higher concentrations of geranylgeranylpyrophosphate can be used or an alternate route of administration can be used, either injection via the tail vein or peritoneal injection. Thus, this EXAMPLE provides guidance for testing how to determine therapeutic or prophylactic dosages of HMGCoA reductase inhibitors.

Effect of C3 exotoxin on bFGF stimulated angiogenesis in the mouse cornea. Mouse corneal pocket assays are carried out using either control pellets, pellets containing 10 ng bFGF, pellets containing 10 ng bFGF plus 5 μM simvastatin or pellets containing bFGF plus

10 μg of C3 exotoxin. If (as expected) a RhoA GTPase affects the anti-angiogenic effect of simvastatin, then C3 exotoxin reverses the angiogenic response to bFGF and mimic the effect of simvastatin. If the inclusion of C3 exotoxin in the pellet has no effect on bFGF stimulated angiogenesis at 5 μg/ml, higher concentrations are used. Alternatively, the toxin is given by injection into the tail vein.

Dependence of VEGF-stimulated angiogenesis in the CAM assay on protein geranylgeranylation. Polymers containing 250 ng VEGF in combination with either 5 μM simvastatin or in the presence of 10 μM GGTI or FTI are implanted. In a second set of assays, meshes containing VEGF plus 5 μM simvastatin and 10 μM geranylgeranylpyrophosphate or farnesylpyrophosphate are used. If direct application of these agents has no effect on angiogenesis, then they can be injected at the appropriate concentrations into the CAM vessels. As in the case of bFGF stimulated angiogenesis in the mouse corneal pocket assay. If (as expected) VEGF-stimulated angiogenesis is dependent on protein geranylgeranylation, then GGTI mimics the effect of simvastatin and geranylgeranylpyrophosphate reverses the effect of simvastatin on angiogenesis. To determine whether VEGF-stimulated angiogenesis is dependent on Rho, the effect of meshes containing VEGF and 5 μg /ml C3 exotoxin on angiogenesis are tested.

Effect of expression of dominant negative Rho mutants on angiogenesis. To further test how members of the Rho family of GTPases are required for the stimulation of angiogenesis, dominant active and dominant negative mutants of RhoA, Rac-1 and Cdc42 is expressed either individually or in combination in CAMs and mouse corneal pockets and their effect on angiogenesis determined. Combinations of dominant activating and dominant negative mutants are not used. Retroviral vectors are used for the expression of genes in both HUVECs and chick cells and adenovirus vectors are used for the expression in HUVECs and in the corneal pocket assay.

Recombinant retrovirus. We have generated constructs of pLNCX retroviruses containing myc-tagged dominant activating L63 RhoA, L61 Rac-1, and L61 Cdc42, and the dominant negative N19 RhoA, N17 Rac-1 and N17 Cdc42 each downstream from a

β-galactosidase and a virus constitutively expressing the tetracycline-controlled transactivator.

Initial tests with HUVECs have demonstrated a 70% infection rate in pLNCX retrovirus expressing a β-galactosidase construct.

Recombinant adenovirus. We have obtained an adenovirus constitutively expressing the tetracycline controlled transactivator (Kalman *et al.*, 10 Mol. Biol. Cell. 1665-83 (1999)). Prior to the initiation of the test, cells are infected with various concentrations of the pLNCX virus expressing a βgal and stained to determine %-infected cells. Cells infected with viruses expressing Rho mutants are stained for c-myc to determine infection rate and expression of a mutant Rho family member. Similar preliminary studies are carried out using the adenoviruses and the infection rates determined by staining for c-myc. In assays in which cells are infected with a combination of viruses expressing several Rho mutants, the expression of the mutant is determined by Western blot analysis of cell extracts using specific antibodies to Rho, Rac-1 or Cdc42. Changes in cellular morphology such as rounding of cells expressing the dominant negative RhoA and the development of spiky tubes in cells expressing the dominant negative Cdc42 are also useful qualitative measurements of transgene expression.

The use of adenovirus to express mutant forms of Rho is as potential hazard. We wear of gloves, lab coats, and respirators during the handling of virus. We handle virally infected cells in either a SterilGARD hood or fume hood until such time as the sample has been inactivated by detergent treatment. Precaution are taken not to allow solutions in contact with the viruses to aerosolize.

The effect of dominant negative Rho mutants on the formation of capillary-like structures in HUVECs cultured on Matrigel. To test the effect of dominant negative Rho mutants on the formation of capillary-like structures in HUVECs cultured on Matrigel, HUVECs are co-infected with pLNCX retroviruses or adenoviruses expressing the Rho mutants and the virus expressing the tetracycline-controlled transactivator, cultured until confluent in the presence of tetracycline, harvested and plated on Matrigel coated plates in the presence or absence of tetracycline and the time course of development of capillary-like structures observed. Alternatively, cells are plated on Matrigel at the time of infection with the mutant containing virus and the transactivator with and without tetracycline and the development of capillary structures determined over 24-48 hr. Cells are infected either with an individual virus or a combination of viruses. Tetracycline controls are included in each assay.

Effect of dominant negative Rho mutants on angiogenesis in the mouse cornea. To determine the effect of dominant negative Rho mutants on bFGF stimulated angiogenesis in an

animal model, mouse corneal pockets are treated with pellets containing 10 ng bFGF. The cornea is anesthetized and adenovirus expressing one of the dominant negative mutants of Rho mutant is dripped onto the corneas. Placing the virus directly on the cornea is necessary, since it is not possible to mix the virus into the pellets because of inactivation by the ethanol necessary for pellet preparation. To maximize viral infection the eye of the anesthetized mouse is maintained in a closed position for various times. The extent of infection and expression of the mutant Rho is determined by staining the corneas for c-myc. It is necessary to vary the concentration of virus and the time of incubation, to maximize the fraction of infected cells as measured by c-myc staining. The absence of an effect of expression of a dominant negative mutant does not constitute proof that Rho does not affect bFGF stimulated angiogenesis. For the same reason it is not possible to assess the effect of expressing more than one mutant at a time.

Effect of dominant negative Rho mutants on angiogenesis in CAM assays. Since the efficiency of infection of corneas by the direct application of adenovirus may not yield levels of infection and expression of Rho mutants sufficient to effect angiogenesis, we use the CAM assay as an alternative animal model to test the effect of Rho in angiogenesis. The CAM has several advantages. (1) The pLNCX retrovirus readily infects chick cells. (2) Although the virus may not survive the preparation of the collagen mesh, it may be injected into the vessels of the chorioallantoic membrane which is more likely to permit their localization in the CAM vasculature.

The CAM assays are designed as described in EXAMPLE 2 above for the corneal pocket assays. The chorioallantoic vessels of CAMs treated with patches containing 250 ng VEGF are injected with the pLNCX retrovirus expressing a dominant negative Rho mutant and the virus expressing the tetracycline-controlled transactivator and the effect on angiogenesis determined after 3 to nine days incubation. CAM assays are carried out according to the protocol.

Effect of dominant activating mutants of Rho on angiogenesis (in vivo assays). This EXAMPLE provides *in vivo* assays to determine the mechanism by which Rho and HMGCoA reductase inhibitors regulate angiogenesis. Using dominant activating Rho mutants the

To test the effect of dominant activating mutants of Rho on angiogenesis in CAMs and the mouse cornea, these assays are carried out as described in EXAMPLE 2 above for the dominant negative mutants, except that growth factors are not included in the pellets and viruses expressing dominant activating mutants of Rho are used.

Culture of endothelial cells on a three-dimensional collagen matrix, a model for endothelial cell invasion and differentiation. To test how dominant activating Rho mutants stimulate angiogenesis *in vitro*, we do not use the Matrigel model, since Matrigel stimulates the formation of capillary-like structures in the absence of additional growth factors. However, one *in vitro* model for testing the induction of angiogenesis is the three-dimensional collagen matrix model. In the collagen matrix model, VEGF induces the invasion of bovine aortic endothelial cells into the collagen matrix and the formation of tube-like structures (Pepper et al., 189 Biochem. Biophys. Res. Commun. 824-31 (1992), Davis & Camarillo, 224 Exp. Cell Res. 39-51 (1996)). The angiogenic effect of VEGF or the expression of Rho mutants can be quantitated by photographing capillary-like structures using a phase contrast microscope focused at a single level beneath the surface monolayer. The total length of all cell cords which penetrate beneath the surface monolayer in each field is determined. This is an excellent model for studying the physiologic consequences of VEGF signaling (Montesano, 22 Eur. J. Clin. Invest. 504-15 (1992); Montesano & Orci, 42 Cell 469-77 (1985); Pepper *et al.*, 111 J. Cell Biol. 743-55 (1990)).

So, in this EXAMPLE, we use both BAECs and HUVECs. Bovine aortic endothelial cells (BAECs) give a robust angiogenic response to VEGF stimulation in this assay. Since the response of BAECs and HUVECs to VEGF is quite similar, BAECs provide a reliable model for these assays. In addition, we determine that inhibition of the cholesterol metabolic pathway in BAECs inhibits angiogenesis as demonstrated for HUVECs. BAECs are cultured on Matrigel and the effects of simvastatin, GGTI, and C3 exotoxin on the formation of capillary-like structures.

Effect of Rho in VEGF-stimulated endothelial cell invasion, migration, and tube formation. We first determine whether Rho regulates the angiogenic response in this model. Since the collagen matrix model measures the ability of VEGF to stimulate endothelial cell invasion and tube formation, we are also testing how HMGCoA reductase inhibitors inhibit angiogenesis by interfering with Rho dependent VEGF signaling.

To test how VEGF signaling is dependent on Rho, BAECs are cultured on a three-dimensional collagen matrix until confluent and incubated for 24 hr in 5% serum cells are transferred to 2% serum and incubated for 24 hr with either sham, 5 μM atorvastatin, 10 μM pravastatin, 5 μM simvastatin, 10 μM GGTI, or 5 μg/ml C3 exotoxin, VEGF is added, the incubation continued for three days and the formation of tubular structures determined. If (as expected) VEGF signaling is dependent on a Rho family member, then based on preliminary data, each of these treatments should interfere with invasion of the collagen matrix and tube formation. We further determine how the effect of simvastatin on invasion of the collagen and tube formation is reversed by incubation of monolayers with simvastatin plus 10 μM geranylgeranylpyrophosphate.

To determine the effect of dominant negative Rho mutants on VEGF stimulation of endothelial cell invasion and tube formation BAECs are infected with adenoviruses expressing dominant negative mutants of RhoA, Cdc42, or Rac-1 and the virus expressing the transactivator and incubated overnight in the presence of tetracycline. Cells are harvested and plated on a thick collagen gel in medium containing 5% serum at a titer sufficient to permit the rapid development of a confluent monolayer. Cells are transferred to 2% serum and incubated either in the presence or absence of tetracycline and the expression of Rho mutants determined by staining for c-myc. VEGF is added and incubation continued for 3 days in the presence and absence of tetracycline and the relative level of tube formation determined. Control plates of uninfected cells incubated with VEGF and tetracycline or with VEGF alone are included. Viral titer are varied to assure adequate levels of expression of the mutant Rho which are monitored by c-myc staining.

The effect of Rho in VEGF-stimulated endothelial cell migration. To further test how HMGCoA reductase inhibitors interfere with angiogenesis by inhibiting VEGF signaling, we determine the effect of Rho in VEGF-stimulated migration in HUVECs. HMGCoA reductase inhibitors interfere with the migration of vascular smooth muscle cells via a process dependent on protein lipidation and that Rho is required for the migration of HUVECs in an *in vitro* wound repair assay (Aepfelbacher *et al.*, 17 Arterioscler. Thromb. Vasc. Biol. 1623-9 (1997), Corsini *et al.*, 33 Pharmacol Res. 55-61 (1996)). [illegible] simvastatin, with 1 nM C3 exotoxin, harvested and plated on FluoroBlock inserts. Cells are incubated with simvastatin and/or other agents added to both the upper and lower

chamber and VEGF is added only to the lower chamber. In a second set of assays, cells are incubated with simvastatin and geranylgeranylpyrophosphate or farnesylpyrophosphate, and the effect on VEGF-stimulated migration is determined. If (as expected) a geranylgeranylated protein affects VEGF-stimulated HUVEC migration, then GGTI, simvastatin, and C3 exotoxin inhibits migration and geranylgeranylpyrophosphate reverses simvastatin inhibition of migration. If (as expected) a Rho GTPase affects VEGF-stimulated migration, then migration is blocked by C3 exotoxin.

Dominant activating Rho mutants mimic the effect of VEGF on tube formation by BAECs in the three-dimensional collagen matrix model and VEGF-stimulated migration of HUVECs. To test how members of the Rho family of GTPases stimulate angiogenesis, HUVECs are infected with adenoviruses expressing dominant activating mutants of RhoA, Cdc42 or Rac-1 and the virus expressing the transactivator and incubated overnight in the presence of tetracycline. Cells are harvested and plated on a thick collagen gel in medium containing 5% serum at a titer sufficient to permit the rapid development of a confluent monolayer. Cells are transferred to medium 2% in serum and incubated either in the presence or absence of tetracycline, and the expression of Rho mutants determined by staining for c-myc. The extent of tube formation is determined after three days in culture. If the expression of the dominant activating Rho mutants results in extensive cell death, we then titrate the expression of the mutant Rho by adding increasing concentrations of tetracycline until a dose is found which permits both cell survival and expression of the mutant Rho as measured by c-myc staining or Western blot analysis. Should a dominant active Rho mutants induce three dimensional tube formation, cells infected with virus expressing this construct are incubated with either 5 μM simvastatin, 10 μM GGTI or 10 μM FTI. Since even the dominant activating Rho mutants require geranylgeranylation and membrane localization for function, simvastatin and GGTI should at least partially reverse the effect of the mutant Rho. The finding that simvastatin and specific inhibitors of the geranylgeranylation of Rho reverse the effect of a dominant activating mutant on three dimensional tube formation would show that the regulation of the cholesterol metabolic pathway interferes with angiogenesis via the inhibition of the posttranslational geranylgeranylation of Rho. The finding that an activated Rho mimics the effect of VEGF on endothelial cell invasion of the collagen matrix and the formation of tube-like structures would be strong evidence that the cellular responses of endothelial cells to VEGF are mediated at least in part by a Rho dependent pathway.

Finally, we determine how dominant activating mutants of Rho stimulate migration of HUVECs. HUVEC monolayers are infected with recombinant adenovirus expressing dominant activating Rho mutants and the transactivator cells are incubated in the presence and absence of tetracycline taking care that the mutated Rho has been expressed and that excessive cell death has not occurred. Cells are labeled and migration determined as described in EXAMPLE 1.

EXAMPLE 7
Rho REGULATES ANGIOGENESIS VIA THE CONTROL OF VEGF SIGNALING

Rho regulates angiogenesis via the control of VEGF signaling at two levels:(1) activation of VEGF receptors,and (2) expression of genes coding for the VEGF ligand and VEGF receptors. In this EXAMPLE, we test how the VEGF-stimulated autophosphorylation of Flt-1 and Flk-1/KDR is dependent on a member of the Rho family of GTPases and that HMGCoA reductase inhibitors interfere with VEGF signaling in part by inhibiting the VEGF-stimulated phosphorylation of Flt-1 and Flk-1/KDR via an effect on the geranylgeranylation of Rho. Thus, this EXAMPLE provides guidance for testing how to determine therapeutic or prophylactic dosages of HMGCoA reductase inhibitors.

Assays provided in this EXAMPLE determine how Rho regulates VEGF signaling. Specifically, we test how Rho regulates VEGF signaling by controlling the VEGF-stimulated auto-phosphorylation of the VEGF receptors Flk-1/KDR and Flt-1, which is required for downstream signaling. Assays provided in this EXAMPLE further test how VEGF signaling is also regulated by Rho at the level of gene expression. Specifically we test how pro-angiogenic stimuli such as thrombin, angiotensin II and hypoxia regulate the expression of VEGF and the VEGF receptors by a Rho dependent pathway and that inhibition of the geranylgeranylation of Rho family members by HMGCoA reductase inhibitors interferes with the induction of VEGF and VEGF receptors.

VEGF stimulation of the tyrosine phosphorylation of Flt-1 and Flk-1/KDR is dependent on the geranylgeranylation of Rho. We first assay to determine whether VEGF-stimulated tyrosine phosphorylation of Flk-1/KDR and Flt-1 are Rho dependent and

[illegible] incubated for 16 hr in medium supplemented with 1% FCS in the absence of growth factors

with 10 μM GGTI or FTI or 5 μg C3 exotoxin. Following a 5 min incubation with 10 ng/ml VEGF, cells are homogenized and the extract immunoprecipitated with anti Flt-1 or anti Flk-1 antibody followed by PAGE and immunoblotting using anti-tyrosine antibody. In 1% serum cell death during the overnight preincubation with simvastatin was less than 20% and basal phosphorylation of Flt-1 and Flk-1/KDR is barely detectable. Our preliminary tests have already indicated that VEGF stimulation of phosphorylation is maximal in less than 5 min.

In a second set of assays, we determine whether the simvastatin inhibition of the phosphorylation of Flk-1/KDR and Flt-1 is reversed by geranylgeranylpyrophosphate. HUVECs are incubated for 16 hr with simvastatin in the presence of either 10 μM geranylgeranylpyrophosphate, or farnesylpyrophosphate and VEGF-stimulated receptor phosphorylation determined. If (as expected) a Rho GTPase is involved in the VEGF stimulation of the phosphorylation of Flt-1 and Flk-1/KDR, then GGTI and C3 exotoxin should inhibit VEGF-stimulated phosphorylation and geranylgeranylpyrophosphate should reverse the inhibitory effect of simvastatin on the phosphorylation of Flt-1 and Flk-1/KDR. We have already shown that simvastatin had no effect on the level of expression of the receptors (*see*, above).

For each assay proposed above, Western blot analysis of an aliquot of cell extract with specific antibody to Flk-1/KDR and Flt-1 are used to determine whether the level of expression of Flk-1/KDR and Flt-1 is altered.

Effect of Rho mutants on the VEGF-stimulated phosphorylation of Flk-1/KDR and Flt-1. To determine the effect of Rho family members in VEGF signaling, the effect of expressing dominant negative and dominant activating mutants of Rho family members on the phosphorylation of Flt-1 and Flk-1/KDR is determined. If (as expected) VEGF-stimulated phosphorylation of Flt-1 and Flk-1 is Rho dependent, then dominant negative mutants interfere with receptor phosphorylation and dominant active mutants might mimic the effect of VEGF. Confluent monolayers of HUVECs are infected, as described in EXAMPLE 6 above, with an adenovirus expressing dominant negative mutants of RhoA, Rac-1, or Cdc42 either individually or in combination and a second virus expressing the tetracycline-controlled transactivator and grown to confluence in tetracycline. Tetracycline is removed and incubation continued for 16 hr followed by a 5 min incubation with VEGF and the effect on the phosphorylation of Flt-1 and Flk-1 determined. Cells are stained for c-myc to determine the expression of Rho.

The effect of HMGCoA reductase inhibitors on the binding of [^{125}I]VEGF and localization of Flk-1/KDR and Flt-1. We determine the number of [^{125}I]VEGF binding sites on the surface of intact cells from control HUVEC cultures or HUVECs incubated 16 hr with 5 μM simvastatin or 10 μM GGTI. After incubation, cells are washed and incubated for 90 min at room temperature in M199 plus 20 mM HEPES, pH 7.4, 0.1% BSA and 100 μg soybean trypsine inihibitor and increasing concentrations of [^{125}I]VEGF in the presence and absence of unlabelled VEGF. The cells are washed and solubilized with 2% SDS in PBS and radioactivity measured. Non-specific binding is subtracted and the specific binding plotted by the method of Schatchard. (Soldi *et al.*, 18 EMBO J. 882-92 (1999)).

An alternative approach is to use FACS analysis to determine whether the receptors are on the cell surface and accessible to the ligand. Cells are rinsed and gently scrapped from the plate, gently resuspended and counted. Cells are incubated with antibodies to either Flt-1 or Flk-1/KDR followed by incubation with a secondary IGG antibody conjugated to FITC. Resuspended cells are subject to FACS analysis.

Rho regulates VEGF signaling by controlling the expression of VEGF, Flt-1 and Flk-1/KDR. In this EXAMPLE, we test how the hypoxia-stimulated expression of VEGF, Flt-1 and Flk-1/KDR is regulated by a member of the Rho family of GTPases and HMGCoA reductase inhibitors are capable of inhibiting the hypoxia-stimulated expression of VEGF and VEGF receptors.

We then test how induction of VEGF, Flt-1 and Flk-1/KDR by angiotensin II, thrombin and hypoxia is dependent on Rho and how HMGCoA reductase inhibit the angiotensin II, thrombin and hypoxia induction of VEGF, Flt-1 and Flk-1/KDR by inhibiting the geranylgeranylation of Rho.

Effect of Rho in the regulation of VEGF, Flt-1 and Flk-1/KDR expression by angiotensin II and thrombin. To test the effect of Rho in the expression of VEGF, Flt-1, and Flk-1/KDR in response to thrombin and angiotensin II, HUVECs are incubated for 16 hr with either GGTI, FTI or C3 exotoxin followed by the addition of thrombin or angiotensin II for 6 hr and the effect on the level of expression of VEGF, Flt-1, and Flk-1/KDR determined. The effect of geranylgeranylpyrophosphate on simvastatin inhibition of thrombin and angiotensin

geranylgeranylpyrophosphate reverses simvastatin inhibition of angiotensin II stimulated VGEF expression.

Effect of Rho in the regulation of VEGF, Flt-1 and Flk-1/KDR expression by hypoxia. To test the effect of Rho in the expression of VEGF, Flt-1 and Flk-1/KDR in response to hypoxia, we use a hypoxia chamber. Cells cultured on 60 mm dishes are incubated for 16 hr in serum supplemented with 1% serum and then transferred to a modulator incubator (Billups-Rothberg) and perfused for 30 min with a mixture of 5% CO_2 and 95% N_2. Under these conditions, the level of O_2 in the chamber is undetectable. The chamber, which is humidified by water in its base, is then sealed and the cells incubated at 37°C for various times and the effect of hypoxia on the expression VEGF, Flt-1, and Flk-1/KDR determined by Western blot analysis. Since HUVECs are sensitive to hypoxia, initially we incubate the cells for various times to determine the incubation time at which cell survival and the expression of VEGF, Flt-1, and Flk-1/KDR are optimal. Alternatively, we will incubate cells under reduced oxygen conditions, 5% O_2, to more closely reproduce hypoxic conditions which might exist *in vivo*. To establish the effect of Rho in the response of VEGF, Flt-1, and Flk-1/KDR to hypoxia, cells are incubated in 1% serum for 24 hr with either simvastatin, GGTI, FTI, or C3 exotoxin, transferred to the hypoxia chamber for 6 hr and the effect of hypoxia on the level of expression of VEGF, Flt-1, and Flk-1/KDR determined. We also determine whether pretreatment of cells with geranylgeranylpyrophosphate and simvastatin reverses the effect of simvastatin on hypoxia-induced expression of VEGF, Flt-1, and Flk-1/KDR.

The effect of dominant negative Rho mutants on angiotensin II-, thrombin-, and hypoxia-induced expression of VEGF, Flt-1 and Flk-1/KDR. In this EXAMPLE, we test how angiotensin II-stimulated VEGF expression is regulated by the activation of a Rho dependent downstream signaling pathway such as ERK, p38 kinase, or the JNK pathway and that HMGCoA reductase inhibitors interfere with angiotensin II-stimulated angiogenesis at least in part by inhibiting this VEGF expression.

HUVECs are infected with the viruses expressing the dominant negative mutants and the virus expressing the tetracycline transactivator in the presence of tetracycline. Cells are transferred to fresh media 1% in serum with and without tetracycline and incubated for 24 hr. The level of expression of the mutant Rhos are determined as described above. Cells will then be incubated for 6 hr with either angiotensin II or thrombin or for 6 hr in the hypoxia chamber

and the level of expression of VEGF, Flt-1 and Flk-1/KDR determined. Dominant negative mutants of RhoA, Rac-1 and Cdc42 differentially inhibit ERK-2, JNK, and p38 kinase.

Determination of Rho dependent pathways involved in angiotensin II, thrombin and hypoxia stimulated induction of VEGF, Flt-1 and Flk-1/KDR expression. In this EXAMPLE, we test how both thrombin and angiotensin II regulate the expression of VEGF, Flt-1, and Flk-1/KDR via a Rho dependent MAP kinase pathway and that HMGCoA reductase inhibitors interfere with thrombin and angiotensin II-stimulated expression of VEGF, Flt-1 and Flk-1/KDR and angiogenesis via the inhibition of the geranylgeranylation of Rho.

Next, we test which of the Rho dependent pathways is involved in the angiotensin II and thrombin induction of VEGF, Flk-1/KDR and Flt-1 expression. Cells are incubated with either thrombin or angiotensin II and the time course and dose dependence of activation of ERK, JNK, and p38 pathways determined using Western blot analysis with commercially available antibodies to the phosphorylated forms of ERK-2, JNK and p38 kinase. The effect of angiotensin II and thrombin on kinase activity is also tested. ERK activity is tested by immunoprecipitating ERK and incubating the precipitated protein with [^{32}P]γATP and myelin binding protein followed by PAGE and autoradiography. JNK activity is tested by immunoprecipitating JNK and incubating the precipitated protein with commercially available c-jun followed by PAGE and Western blot analysis with anti-phos-jun antibody. p38 MAP kinase is assayed by immunoprecipitating p38 MAP kinase and incubating the precipitated protein with ATF_2 and [^{32}P]γATP followed by PAGE and autoradiography.

We use a combination of dominant negative mutants and specific inhibitors of each pathway, to determine which pathway is involved in the induction of VEGF, Flt-1 and Flk-1/KDR. The ERK pathway is inhibited by PD 98059, p38 kinase pathway by SB203580 and a dominant negative p38kinase and JNK/SAPK by a dominant negative JNK. The cDNAs coding for dominant negative mutants of JNK and p38 kinase is from Chen *et al.*, 271 J. Biol. Chem. 31929-36 (1996). (We have already generated myc-tagged cDNAs under the control of the tetracycline repressor for the dominant active and dominant negative mutants of RhoA, Rac-1 and Cdc42.) We then generate similar constructs from the dominant negative mutants of p38 kinase and JNK. These constructs are used to generate recombinant adenoviruses

thrombin for 7-15 min and with increasing concentrations of the ERK kinase inhibitor PD

98059 or p38 kinase inhibitor SB203580 and the phosphorylation of ERK and p38 kinase determined as described above. Cells incubated for 24 hr in 1% serum are incubated for 6 hr with either thrombin or angiotensin II either under control conditions or with 30 μM PD 98059 or 10 μM SB203580 and the expression of VEGF, Flt-1, and Flk-1/KDR determined by Western blot analysis. To test dominant negative mutants of Rho, cells are infected with viruses expressing either the dominant negative JNK or the dominant negative p38 kinase and the virus expressing the transactivator and incubated for 24 hr in 1% serum plus tetracycline. Tetracycline is removed and incubation continued until expression of the mutant JNK or p38 kinase are demonstrated by staining with c-myc antibody, 9E10 or Western blot analysis of cell extracts with monoclonal antibody 9E10. Cells are further incubated for 6 hr with or without tetracycline and either thrombin or angiotensin II and the expression of VEGF, Flt-1, Flk-1/KDR determined.

EXAMPLE 8
THE POTENTIATION OF VEGF SIGNALING BY INTEGRINS IS DEPENDENT ON A Rho GTPase.

HMGCoA reductase inhibitors interfere with VEGF signaling by disrupting the cross-talk between VEGF and integrin signaling. Assays provided in this EXAMPLE test how the interaction between VEGF and integrin signaling is dependent on a member of the Rho family of GTPases and that HMGCoA reductase inhibitors interfere with VEGF signaling and angiogenesis by disrupting the interaction between VEGF and integrin signaling.

VEGF-stimulated phosphorylation of FAK is dependent on Rho. The assays in this EXAMPLE are based on data presented in EXAMPLE 2, which demonstrate that simvastatin interferes with VEGF-stimulated tyrosine phosphorylation of FAK, but has no effect on the expression of FAK. That assay was carried out at a single concentration of simvastatin. To expand upon that data point and to provide guidance for determining a range of appropriate therapeutic or prophylactic dosages, we first determine the concentration dependence of simvastatin inhibition of VEGF-stimulated FAK phosphorylation. HUVECs are incubated for 24 hr in 1% serum with increasing concentrations of simvastatin followed by a 5 min incubation with VEGF. Cell extracts are immunoprecipitated with anti-FAK antibody followed by PAGE and Western blot analysis with anti-phosphotyrosine antibody. An aliquot of each cell extract is subjected to Western blot analysis with anti-FAK antibody to determine the effect of simvastatin on FAK expression.

HUVECs are cultured in the presence of GGTI, FTI or C3 exotoxin, followed by a 5 min incubation with VEGF and tyrosine phosphorylation of FAK determined. To determine whether geranylgeranylpyrophosphate, the substrate for geranylgeranyltransferase, reverses simvastatin inhibition of VEGF stimulated FAK phosphorylation, cells are cultured with simvastatin with or without either geranylgeranylpyrophosphate or farnesylpyrophosphate followed by a 5 min incubation with VEGF and the phosphorylation of FAK determined. Then, tests are carried out to determine which members of the Rho family of GTPases is involved.

Cells are infected with adenovirus expressing the dominant negative mutants of RhoA, Rac-1, or Cdc42, either individually or in combination and the virus expressing the transactivator in the presence of tetracycline. Once cells are confluent and infection is complete, fresh medium is added with or without tetracycline and incubation continued until expression of the myc-tagged Rho mutant can be detected by immunostaining. Cells are incubated for 5 min with VEGF and the phosphorylation of FAK determined. Since the phosphorylation of FAK is transient, a dominant activating Rho mutant should not have an effect on the steady state level of FAK phosphorylation, but may potentiate VEGF-stimulated FAK phosphorylation. Cells are infected as described in EXAMPLE 6 (above), with the dominant activating mutants of Rho family members and the phosphorylation of FAK in response to incubation with increasing concentrations of VEGF-determined in control cells (tetracycline) and cells expressing the Rho mutant the level of FAK phosphorylation determined.

VEGF-stimulated invasion and tube formation by BAECs in a three-dimensional collagen matrix is mediated in part by FAK. We then test how VEGF signaling is dependent on FAK using the collagen matrix assay as a measure of VEGF signaling. If (as expected) VEGF stimulation of BAEC invasion and tube formation in the collagen matrix model for angiogenesis is regulated by a member of the Rho family of GTPases, we then use dominant activating and dominant negative mutants of FAK to determine whether Rho dependent VEGF signaling is dependent on the activation of FAK. We determine whether a dominant negative mutant of FAK interferes with VEGF-stimulated cell migration and tube formation and

[illegible] dominant negative mutant of FAK in the presence of tetracycline. Cells are harvested and

plated on a three-dimensional collagen matrix and grown to confluence in 5% serum plus tetracycline. Medium is removed and replaced with fresh medium containing 2% serum with or without tetracycline and incubation continued until myc staining demonstrates the expression of the mutant FAK. VEGF is added to the medium and incubation continued for 4 days and the extent of formation of capillary-like structures determined. If (as expected) a dominant negative FAK mutant inhibits VEGF signaling, then the Rho dependent VEGF stimulation of FAK phosphorylation, which was inhibited by simvastatin and C3 exotoxin, has an important effect in VEGF signaling.

In converse assays, BAECs are infected with the dominant activating FAK mutant and the virus expressing the transactivator and incubated until confluent in tetracycline, harvested and plated on the three dimensional collagen in 5% serum plus or minus tetracycline. GGTI, simvastatin or C3 exotoxin are added and incubation continued for 24 hr. Since the dominant activating FAK mutant could induce cell migration, we optimize the time of expression of the dominant activating FAK mutant and the time of pretreatment with simvastatin and GGTI. Cells expressing the dominant activating mutant FAK and treated with either GGTI or simvastatin are incubated either alone or with VEGF and the effect on invasion of the three-dimensional collagen matrix and tube formation determined.

If a dominant activating FAK mutant induces VEGF-stimulated tube formation in cells treated with GGTI, simvastatin or C3 exotoxin, then these results show that FAK affects Rho dependent VEGF signaling and angiogenesis and that FAK is downstream from Rho in this cross-talk signaling pathway.

Effect of FAK in VEGF-stimulated phosphorylation of Flt-1 and Flk-1/KDR. To determine the effect of FAK in VEGF stimulated phosphorylation of Flt-1and Flk-1/KDR, monolayers of HUVECs cultured in 1% serum are infected with recombinant adenovirus expressing the dominant negative FAK mutant and the transactivator in the presence of tetracycline, the cells transferred to fresh medium in the presence and absence of tetracycline, VEGF added for 5 min. and the level of phosphorylation of Flt-1and Flk-1/KDR determined.

We determine whether the dominant activated FAK mutant reverses the effect of HMGCoA reductase inhibitors, GGTI, or C3 exotoxin on the VEGF-stimulated tyrosine phosphorylation of Flt-1and Flk-1/KDR. Cells infected with the adenovirus expressing the dominant activating FAK mutant and the transactivator as described above are incubated for 24 hr with no additions, with simvastatin, GGTI, or C3 exotoxin followed by a 5 min

incubation with VEGF and the tyrosine phosphorylation of Flt-1and Flk-1/KDR determined. This assay shows whether an activated FAK is capable of potentiating VEGF stimulated phosphorylation of Flt-1and Flk-1/KDR and whether the phosphorylation of FAK is necessary for the phosphorylation of Flt-1and Flk-1/KDR.

The dominant inhibiting FAK mutant is the carboxy-terminal of $pp125^{FAK}$ designated $pp41/43^{FRNK}$, which interferes with the binding of $pp125^{FAK}$ (Richardson & Parsons, 380 Nature 538-40 (1996)). The dominant activating mutant FAK is a transmembrane anchored chimeric receptor kinase consisting of the T cell CD2 receptor ligated to $pp125^{FAK}$ with constitutively activated kinase activity (Chan *et al.*, 269 J. Biol. Chem. 20567-74 (1994), Frisch *et al.*, 134 J. Cell Biol. 793-9 (1996)).

Effect of Rho in integrin potentiation of VEGF signaling. To determine the effect of cell adhesion on VEGF-stimulated phosphorylation of VEGF receptors, cells are cultured for 24 hr in 1% serum on plastic dishes coated with gelatin under control conditions or in the presence of either GGTI, simvastatin, or C3 exotoxin. Cells grown under all 4 conditions will either be left adherent to the plate or detached by treatment with cold PBS plus 2 mM EGTA followed by suspension in fresh warm medium. Cells in suspension and adherent cells are incubated for 10 min with 10 ng/ml VEGF and the level of tyrosine phosphorylation of Flt-1 and Flk-1/KDR compared. The effect of cell attachment and inhibition of the geranylgeranylation of Rho on the relative level of expression of Flt-1 and Flk-1/KDR is compared to that in cells in suspension by subjecting aliquots of cell extracts to PAGE followed by Western blot analysis with antibodies to Flt-1 and Flk-1/KDR. These tests determine how the potentiation of tyrosine phosphorylation of Flt-1 and Flk-1/KDR in adherent cells is dependent on the geranylgeranylation of a member of the Rho family of GTPases and how this Rho-dependent stimulation affects the level of expression of VEGF receptors.

Ligand specificity for integrin potentiation of VEGF-stimulated phosphorylation of Flt-1 and Flk-1/KDR. To determine how a specific matrix protein affects the Rho dependent potentiation of VEGF-stimulated tyrosine phosphorylation of Flt-1 and Flk-1/KDR, HUVECs

[illegible] simvastatin or C3 exotoxin, followed by a [illegible] incubation in 10 ng/ml VEGF. Cells are harvested and the level of phosphorylation and expression of Flt-1and Flk-1/KDR

determined as described above. Prior studies have demonstrated that plating of cells on vitronectin results in the largest potentiation of VEGF-stimulated phosphorylation of Flk-1/KDR (Soldi *et al.*, 18 EMBO J. 882-92 (1999)). Since poly-L-lysine does not significantly stimulate integrin signaling and hence does not potentiate VEGF-stimulated phosphorylation of Flt-1and Flk-1/KDR, the use of poly-L-lysine provides a useful baseline for VEGF-stimulated phosphorylation of the receptors in the absence of integrin signaling. For assays using ploy-L-lysine, cells are incubated for 2 hr with 1 μM cycloheximide and 1 hr with 1 μM monensin to block the synthesis of extracellular matrix prior to incubation with VEGF. Cells are detached in cold PBS containing 2 mM EGTA then plated on poly-L-lysine or fibronectin for one hour and the effect of VEGF on the phosphorylation of Flt-1and Flk-1/KDR compared with and without pretreatment with GGTI, simvastatin or C3 exotoxin. This adhesion assay is used to determine the specificity of integrins for the potentiation of VEGF-stimulated phosphorylation of Flt-1 and Flk-1/KDR. Cells in suspension are incubated with increasing concentrations of antibodies against α_v, β_3, β_1, α_2, and α_5 at 4°C for 20 min and then plated on vitronectin for 1 hr, treated with VEGF and the effect on the tyrosine phosphorylation of Flt-1 and Flk-1/KDR determined. Alternatively, adherent cells cultured on vitronectin are preincubated with antibodies to integrin subunits, washed, and then incubated with VEGF and the level of tyrosine phosphorylation of Flt-1 and Flk-1/KDR determined.

To determine the effect of members of the Rho family of GTPases in the integrin mediated potentiation of VEGF receptor phosphorylation, we compare the level of VEGF-stimulated phosphorylation of Flt-1 and Flk-1/KDR in cells cultured on matrix proteins and infected with adenovirus expressing dominant negative mutants of RhoA, Rac-1 and Cdc42 either singly or in combination. Cells expressing the dominant negative mutants plated on vitronectin or poly-L-lysine treated dishes are incubated with VEGF and the effect of the Rho mutant on tyrosine phosphorylation and expression of Flt-1 and Flk-1/KDR determined. Since the expression of $\alpha_v\beta_3$ is regulatable, we test by Western blot analysis to determine whether the decreased response of Flt-1 and Flk-1/KDR phosphorylation to stimulation of the extracellular matrix is due to an effect of simvastatin, GGTI, or C3 exotoxin on the expression of $\alpha_v\beta_3$. The relative adhesion of cells under all of the above growth conditions is compared by growing cells in a 96-well plate under each condition, fixing the cells followed by staining with crystal violet and reading the absorbance at 540 nm in a microtiter plate reader.

The effect of Rho on the localization of VEGF receptors. It has previously been reported that integrin potentiation of VEGF-stimulated phosphorylation of Flk-1/KDR was not associated with an increase in the expression of Flk-1/KDR (Soldi *et al.*, 18 EMBO J. 882-92 (1999)). However, the absence of a change in total expression of Flt-1 and Flk-1/KDR does not rule out the possibility that inhibition of the geranylgernylation of Rho effects integrin potentiation of VEGF stimulated phosphorylation of Flt-1 and Flk-1/KDR by decreasing the availability of the receptors to VEGF at the cell surface. This possibility is ruled out by determining the effect of treatment of HUVECs cultured on extracellular matrix proteins with simvastatin, GGTI, or C3 exotoxin on the binding of [^{125}I]VEGF to intact cells and the binding of anti-Flt-1 and Flk-1/KDR antibody to cells measured by FACS analysis.

Effect of FAK in integrin potentiation of VEGF-stimulated receptor phosphorylation. Although $\alpha_v\beta_3$ in the presence of VEGF is associated with the Flt-1/KDR complex, no data have previously been presented which address the mechanism by which integrin signaling regulates VEGF-stimulated phosphorylation of VEGF receptors. To test how integrins communicate with the VEGF receptor through FAK, we determine the effect of expression of FAK mutants on vitronectin potentiation of VEGF-stimulated phosphorylation of Flt-1 and Flk-1/KDR in HUVECs. Cells infected with recombinant adenoviruses expressing dominant negative FAK and the adenovirus expressing the transactivator cultured in the absence or absence of tetracycline to permit the expression of the mutant FAK are cultured for 24 hr on vitronectin or poly-L-lysine, and incubation continued for 10 min in the presence or absence of VEGF and the level of phosphorylation of Flt-1 and Flk-1/KDR determined. An effect of a dominant negative FAK mutant on vitronectin potentiation of VEGF signaling would support the conclusion that FAK affects mediating this potentiation. If (as expected) the dominant negative mutant of FAK does not interfere with vitronectin potentiation of VEGF-stimulated phosphorylation of the VEGF receptors, integrins might communicate with VEGF signaling via a FAK independent pathway. If the dominant negative FAK mutant completely inhibits VEGF stimulated phosphorylation of FAK, then the dominant negative FAK interferes both with the integrin independent VEGF stimulation of Flt-1 and Flk-1/KDR phosphorylation and with the integrin dependent potentiation of VEGF stimulated phosphorylation of Flt-1 and

reductase inhibitors interfere with integrin potentiation of VEGF signaling, we then test the

effect of FAK in mediating the cross-talk between integrins and VEGF by determining whether a dominant activating FAK mutant is capable of reversing simvastatin, GGTI or C3 exotoxin inhibition of integrin potentiation of VEGF signaling. HUVECs expressing a dominant activating FAK mutant cultured on fibronectin are treated for 24 hr under control conditions, or with simvastatin, GGTI or C3 exotoxin followed by a 10 min incubation with or without VEGF and the phosphorylation of Flt-1 and Flk-1/KDR determined. If (as expected) the dominant activating FAK mutant reverses the effect of Rho inactivation, then the cross-talk between integrins and VEGF is dependent on a Rho family member and that it is mediated through FAK.

If (as expected) a dominant activating FAK mutant reverses the effect of GGTI, simvastatin or C3 exotoxin on VEGF signaling in BAECs cultured on a collagen matrix, then FAK may potentiate VEGF stimulated phosphorylation of Flt-1 and Flk-1/KDR. Hence, a dominant negative FAK mutant interferes with VEGF-stimulated phosphorylation of Flt-1and Flk-1/KDR and a dominant activating mutant FAK potentiates VEGF-stimulated phosphorylation of Flt-1and Flk-1/KDR.

Integrins potentiate VEGF signaling by inducing the expression of VEGF. In assays provided in this EXAMPLE, we test how integrins induce the expression of VEGF in HUVECs via a Rho-dependent MAP kinase pathway. HUVECs cultured on either vitronectin, fibronectin or gelatin in the presence or absence of antibodies to either α_v, β_3, β_1, α_2, or α_5, and the expression of VEGF determined by Western blot analysis. If (as expected) an effect of integrins on the expression of VEGF is determined, we then use simvastatin, GGTI, C3 exotoxin and dominant negative mutants to determine how the increased expression of VEGF is Rho dependent and the expression of FAK mutants to determine whether it is dependent on FAK. Finally, we use inhibitors of the ERK and p38 kinase pathways and dominant negative mutants of JNK and p38 kinase, as described above, to determine which of these pathways affects the integrin stimulation of VEGF expression.

EXAMPLE 9
THE EFFECT OF SIMVASTATIN ON THE EXPRESSION OF VEGF OR VEGF RECEPTORS AND NEW BLOOD VESSEL FORMATION IN THE ATHEROSCLEROTIC PLAQUES OF CHOLESTEROL-FED OR ANGIOTENSIN II TREATED ApoE-/- MICE

In this EXAMPLE, we assay *in vivo* for the regulation of VEGF signaling by Rho and HMGCoA reductase inhibitors, to show that the inhibitors have a clinical effect in the

therapeutic effect of HMGCoA reductase inhibitors in atherosclerosis. We test how HMGCoA reductase inhibitors interfere with the growth of atherosclerotic lesions by inhibiting VEGF signaling and angiogenesis in the ApoE-/- mouse.

HMGCoA reductase inhibitors inhibit the expression of VEGF, Flt-1 and Flk-1/KDR and interfere with plaque formation and growth by inhibiting angiogenesis in cholestero-fed ApoE-/- mice. ApoE-/- mice are cholesterol-fed for 12 weeks prior to initiation of simvastatin treatment. The correlation between plaque development, plaque size, and the expression of VEGF, Flt-1, and Flk-1/KDR are determined initially. The effect of simvastatin on expression VEGF, Flt-1, and Flk-1/KDR is tested. The new blood vessel formation in atherosclerotic plaques is then correlated with effects on plaque size and growth.

Male ApoE-/- mice 6 to 8 weeks of age are fed a 0.15% cholesterol diet. At 20 weeks of age 10 animals are sacrificed to evaluate baseline extent of atherosclerosis. The remaining animals are divided into 2 groups and treated for 16 weeks. Group 1 continues with the same diet, but the feed for the animals in group 2 contains simvastatin, for a total daily dose of 30 mg/kg. In previous studies, the anti-angiogenic agents TPN-470 and endostatin exerted the most significant effects when administered between weeks 20 and 36 (Moulton *et al.*, 99 Circulation 1726-32 (1999); Shepherd *et al.*, 333 N. Engl. J. Med. 1301-7 (1995)). At 36 weeks, the animals are euthanized and a sample of blood taken for determination of serum cholesterol. The heart and aorta are perfused with 2% paraformaldehyde for fixation and the heart and portions of the descending aorta embedded in parafin, sectioned, digested with protease XXIV, and incubated with either a rabbit polyclonal anti-von Willebrand Factor antibody for staining of blood vessels or rat monoclonal anti-mouse CD31 for staining of endothelial cells. For VEGF, a rabbit polyclonal antibody raised against the 20 amino-terminal residues of human VEGF are used (Santa Cruz). This antibody neutralizes VEGF activity and reacts specifically with native and denatured VEGF by Western blot. For the identification of Flt-1 and Flk-1/KDR, rabbit polyclonal antibodies are used (Santa Cruz). Primary antibodies are detected with a secondary antibody conjugated to horseradish peroxidase. Intimal vessels are detected under high power magnification and counted when both an endothelial nucleus and lumen can be seen and when the vessel can be seen in an adjacent section.

[illegible] digital camera and measured with the Leica Q500 MC image-analysis program. Total surface area containing

VEGF$^+$ cells is quantified by using computer-aided planimetry and expressed as a percentage of total surface area of intima. In addition, the total surface occupied by VEGF$^+$ endothelial cells, the VEGF$^+$ EC area, is quantified in a similar manner and expressed as a percentage of the total surface area occupied by endothelial cells, as shown by von Willebrand factor staining. The luminal surface area occupied by von Willebrand factor staining is also estimated as a percent of the whole luminal surface area. The signal from Flt-1 and Flk-1/KDR may be more difficult to quantitate since it has been reported to be less intense than that for VEGF. Peritoneal macrophages from mice in each group are harvested from peritoneal fluid and of ApoE-/- mice and the level of VEGF determined by Western blot analysis.

Angiotensin II-treatment induces the expression of VEGF, Flt-1, and Flk-1/KDR in parallel with increasing neo-vascularization and plaque size and these effects of angiotresin II are inhibited by HMGCoA reductase inhibitors in angiotensin II-treated the ApoE-/- mice. This EXAMPLE provides guidance for testing how the anti-angiogenic effect affects limiting the growth and size of atherosclerotic plaques in the ApoE-/- mouse. In assays provided in this EXAMPLE, we test how angiotensin II stimulates the expression of VEGF, Flt-1, and Flk-1/KDR and the development of new blood vessels in atherosclerotic lesions induced by chronic administration of angiotensin II to ApoE-/- mice. Male ApoE-/- mice 6-8 weeks old are fed a normal diet and divided into 4 groups. Two are given a daily intraperitoneal injection either placebo or some ml of 10^{-7} M angiotensin II daily. A third group is given the same dose of angiotensin II plus losartan, an angiotensin II type 1 receptor blocker. A fourth group receives daily intraperitoneal injections of angiotensin II plus simvastatin. Mice from the control group are sacrificed at the initiation of treatment to establish a baseline.

To determine the time course of development of increased expression of VEGF, Flt-1 and Flk-1 and new blood vessel formation animals are sacrificed at six week intervals following the initiation of angiotensin II-treatment until week 30. Aortas analyzed as described in this EXAMPLE (above) for plaque density, microvessel formation, VEGF, Flt-1, and Flk-1/KDR staining. Since ApoE-/- deficient mice develop atherosclerotic lesions spontaneously on a normal diet, the inclusion of time points at 6 week intervals makes it likely that we are including the time period during which the potentiation of lesion formation by angiotensin II is significantly different than that of control animals. Peritoneal macrophages from mice in each group are harvested from peritoneal fluid and of angiotensin II-treated and

control ApoE-/- mice. The level of VEGF is determined by Western blot analysis. Data are analyzed by ANOVA and Student's t test.

In the *in vitro* and *in vivo* models for angiogenesis described in this application, HMGCoA reductase inhibitors interfere with angiogenesis in response to extracellular matrix, VEGF and bFGF. This effect can reasonably considered to be due to the interference of HMGCoA reductase inhibitors with VEGF signaling at the level of VEGF receptor activation and expression of VEGF and VEGF receptors. The assays outlined in this EXAMPLE provide guidance for testing how angiogenesis is dependent on the geranylgeranylation of proteins of the Rho family of GTPases. Hence, the assays of this EXAMPLE establish a new relationship between cholesterol metabolism and angiogenesis.

The assays of this EXAMPLE provide guidance for the clinical relevance of HMGCoA reductase inhibitors, showing how HMGCoA reductase inhibitors inhibit the development of atherosclerotic plaques and the accompanying formation of new blood vessels. Thus, the assays of this EXAMPLE provide new insights into pathogenesis and treatment.

The foregoing description has been presented only for the purposes of illustration and is not intended to limit the invention to the precise form disclosed, but only by the claims appended hereto.

CLAIMS

WE CLAIM:

1. A method for reducing angiogenesis in the tissue of a host, comprising:
administering a therapeutically effective amount of an HMGCoA reductase inhibitor to the tissue of a host, wherein the administration reduces angiogenesis in the tissue.

2. The method of claim 1, wherein the host has a disease selected from the group consisiting of rheumatoid arthritis, diabetic retinopathy, psoriasis, a primary tumor, a metastatic tumor, or atherosclerosis.

3. The method of claim 1, wherein the HMGCoA reductase inhibitor interferes with the vascularization of atherosclerotic plaques.

4. The method of claim 1, wherein the HMGCoA reductase inhibitor is selected from the group consisiting of simvastatin, pravastatin, lovastatin, atorvastatin, fluvastatin, and cerevastatin.

5. The method of claim 1, wherein the dosage is a standard therapeutic dosage.

6. The method of claim 1, wherein the dosage is a higher than standard therapeutic dosage.

7. The method of claim 1, wherein the dosage is a lower than standard therapeutic dosage.

8. A method for preventing angiogenesis angiogenesis in the tissue of a host, comprising:
administering a prophylactically effective amount of an HMGCoA reductase inhibitor to the tissue of a host, wherein the administration prevents angiogenesis in the tissue.

9. The method for birth control, comprising:

administering an effective amount of an HMGCoA inhibitor to the tissue of a female host, wherein the administration prevents uterine vascularization.

10. A method for identifying an inhibitor of angiogenesis, comprising the steps of:

(a) assaying the cellular response of endothelial cells to an angiogenic factor;

(b) assaying the cellular response of endothelial cells to an angiogenic factor in the presence of an HMGCoA reductase inhibitor, wherein the presence of the HMGCoA reductase inhibitor inhibits the cellular response of the endothelial cells;

(c) assaying the cellular response of endothelial cells to an angiogenic factor in the presence of a test compound; and

(d) comparing the cellular response of endothelial cells from step (a) with the cellular response of endothelial cells from step (b) and the cellular response of endothelial cells from step (c), wherein an inhibition of the cellular response of endothelial cells from step (c) as compared with the cellular response of endothelial cells from step (a) identifies the test compound as an inhibitor of angiogenesis.

11. A method for identifying an inhibitor of angiogenesis, comprising the steps of:

(a) assaying the activity of small GTP-binding protein activity from an endothelial cell;

(b) assaying the activity of small GTP-binding protein activity from an endothelial cell that has been contacted with an HMGCoA reductase inhibitor, wherein the contact by the HMGCoA reductase inhibitor inhibits the activity of small GTP-binding protein activity in the endothelial cell;

(c) assaying the activity of small GTP-binding protein activity from an endothelial cell that has been contacted with a test compound; and

activity from an endothelial cell from step (b) and the activity of small GTP-binding protein activity from an endothelial cell from step (c), wherein an

inhibition of the activity of small GTP-binding protein activity from an endothelial cell from step (c) as compared with the activity of small GTP-binding protein activity from an endothelial cell from step (a) identifies the test compound as an inhibitor of angiogenesis.

12. A method for identifying an inhibitor of angiogenesis, comprising the steps of:

(a) assaying the formation of organized structures *in vitro* by endothelial cells;

(b) assaying the formation of organized structures *in vitro* by endothelial cells in the presence of an HMGCoA reductase inhibitor, wherein the presence of the HMGCoA reductase inhibitor inhibits the formation of organized structures *in vitro* by endothelial cells;

(c) assaying the formation of organized structures *in vitro* by endothelial cells in the presence of a test compound; and

(d) comparing the formation of organized structures *in vitro* by endothelial cells from step (a) with the formation of organized structures *in vitro* by endothelial cells from step (b) and the formation of organized structures *in vitro* by endothelial cells from step (c), wherein an inhibition of the formation of organized structures *in vitro* by endothelial cells from step (c) as compared with the formation of organized structures *in vitro* by endothelial cells from step (a) identifies the test compound as an inhibitor of angiogenesis.

13. A method for identifying an inhibitor of angiogenesis, comprising the steps of:

(a) assaying the formation of blood vessels *in vivo*;

(b) assaying the formation of blood vessels *in vivo* in the presence of an HMGCoA reductase inhibitor, wherein the presence of an HMGCoA reductase inhibitor inhibits the formation of blood vessels;

(c) assaying the formation of blood vessels *in vivo* in the presence of a test compound; and

(d) comparing the formation of blood vessels in step (a) with the formation of blood vessels in step (b) and the formation of blood vessels in step (c), wherein an inhibition of the formation of blood vessels in step (c) as compared with the

formation of blood vessels in step (a) identifies the test compound as an inhibitor of angiogenesis.

14. An article of manufacture, comprising packaging material and a primary reagent contained within said packaging material, wherein:

(a) the primary reagent is an HMGCoA reductase inhibitor; and

(b) the packaging material comprises a label which indicates that the primary reagent can be used for reducing angiogenesis in the tissue of a host.

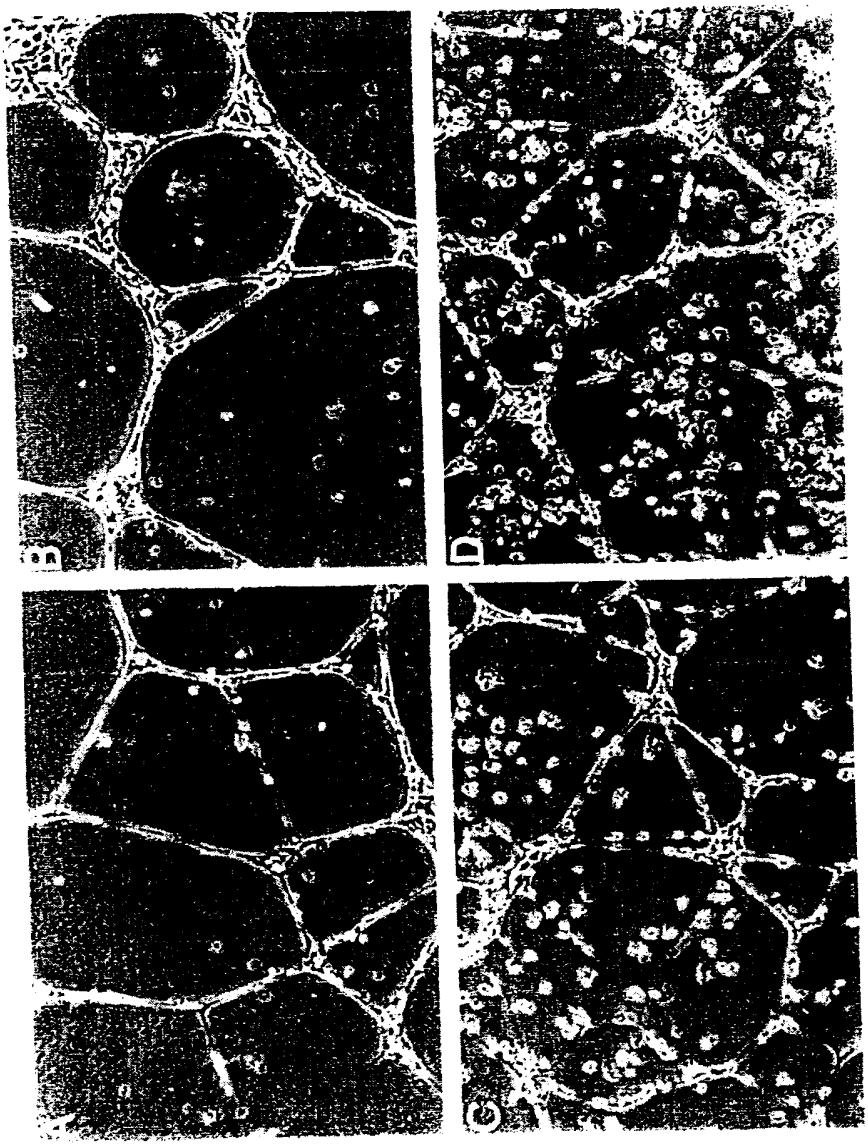


Fig.1 A-D

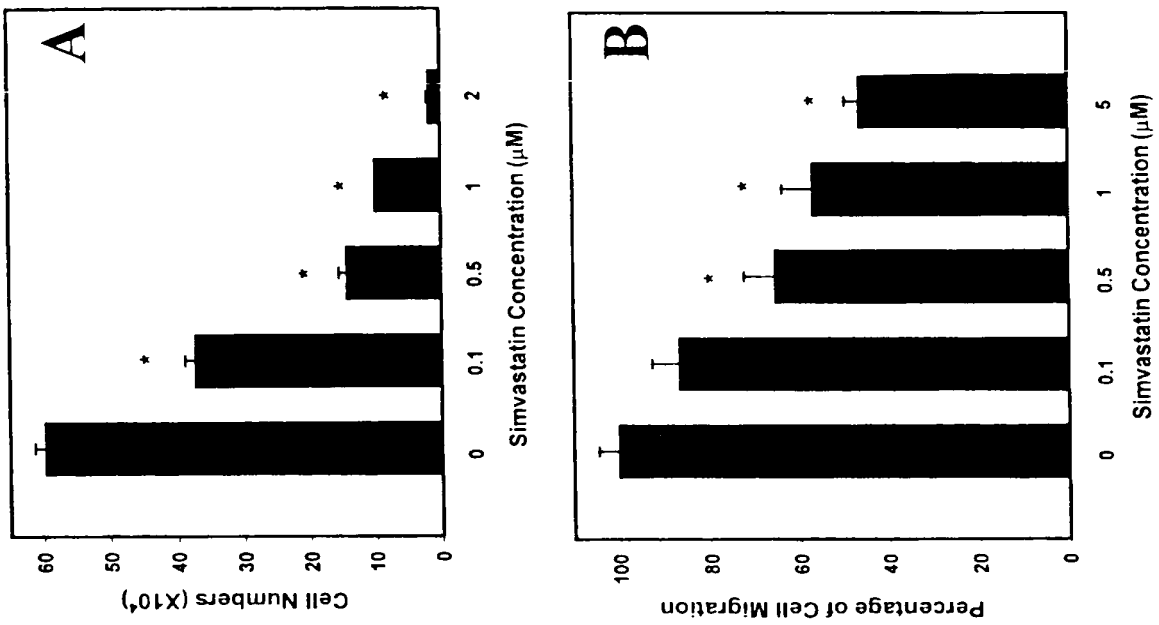

A
Cell Numbers (X10⁴)
0
10
20
30
40
50
60
Simvastatin Concentration (μM)
0
0.1
0.5
1
2
B
Percentage of Cell Migration
0
20
40
60
80
100
Simvastatin Concentration (μM)
0
0.1
0.5
1
5

FIG 2 A-B

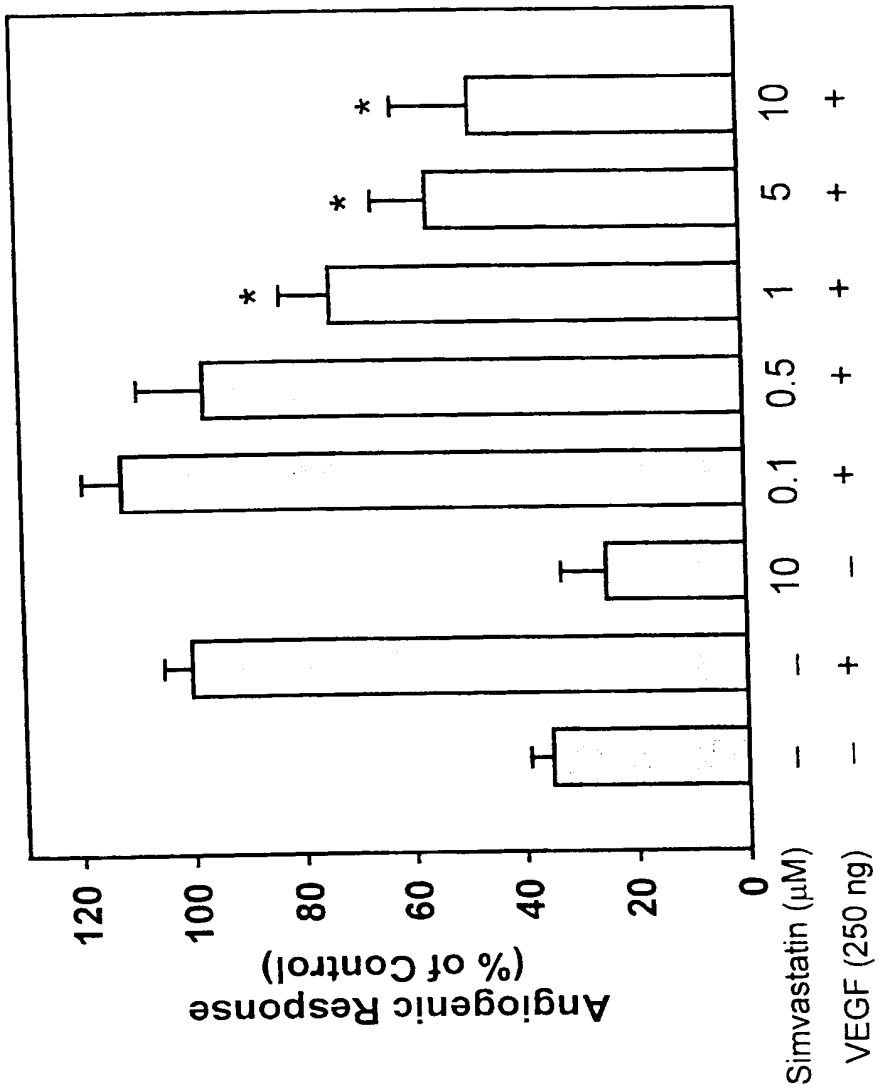


Fig. 3

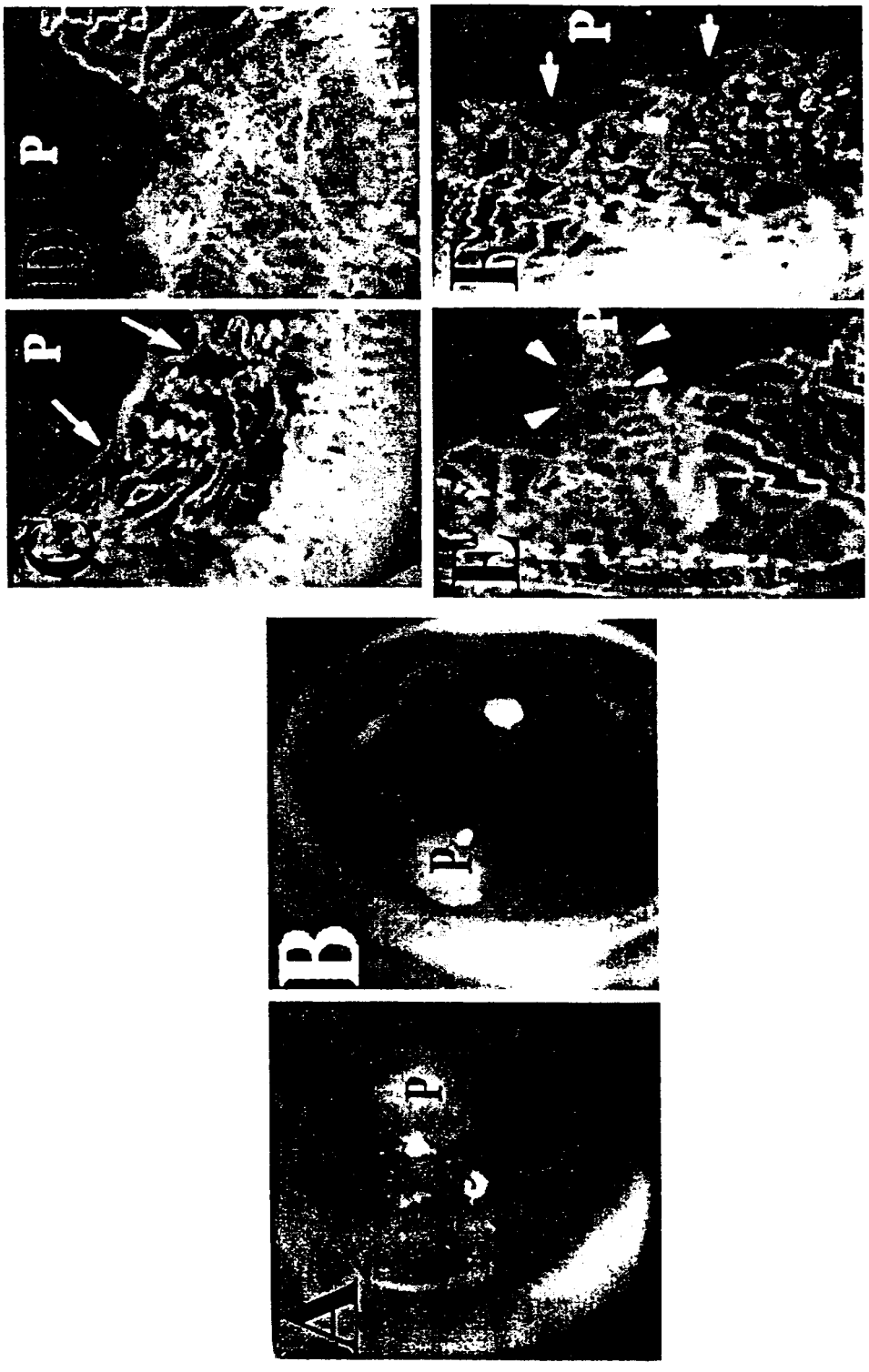


Fig.4 A-F

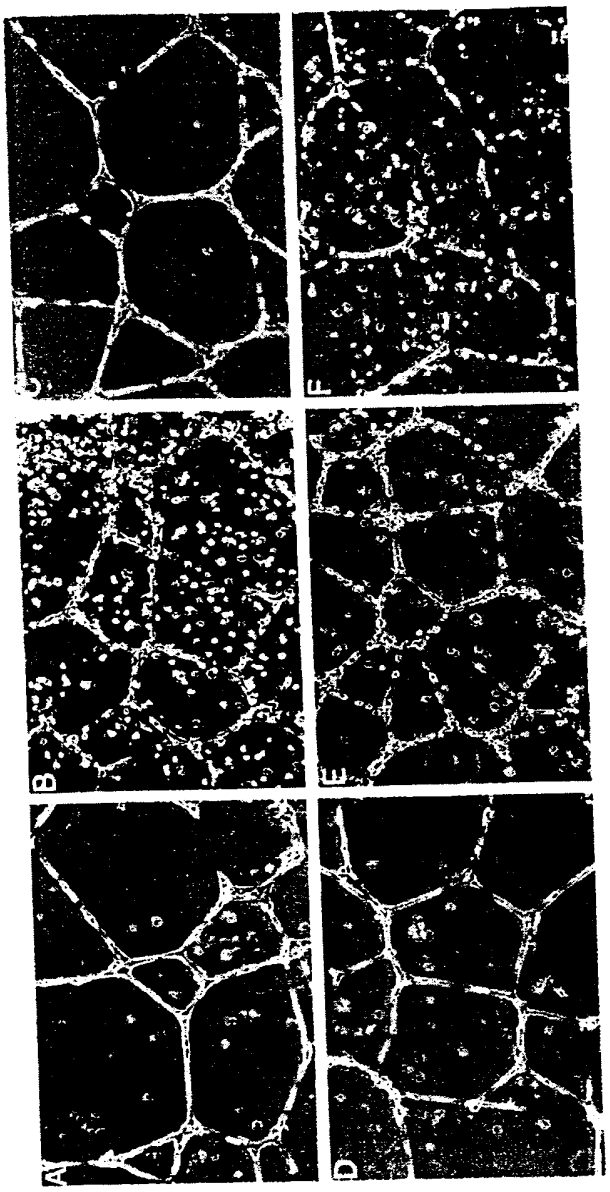
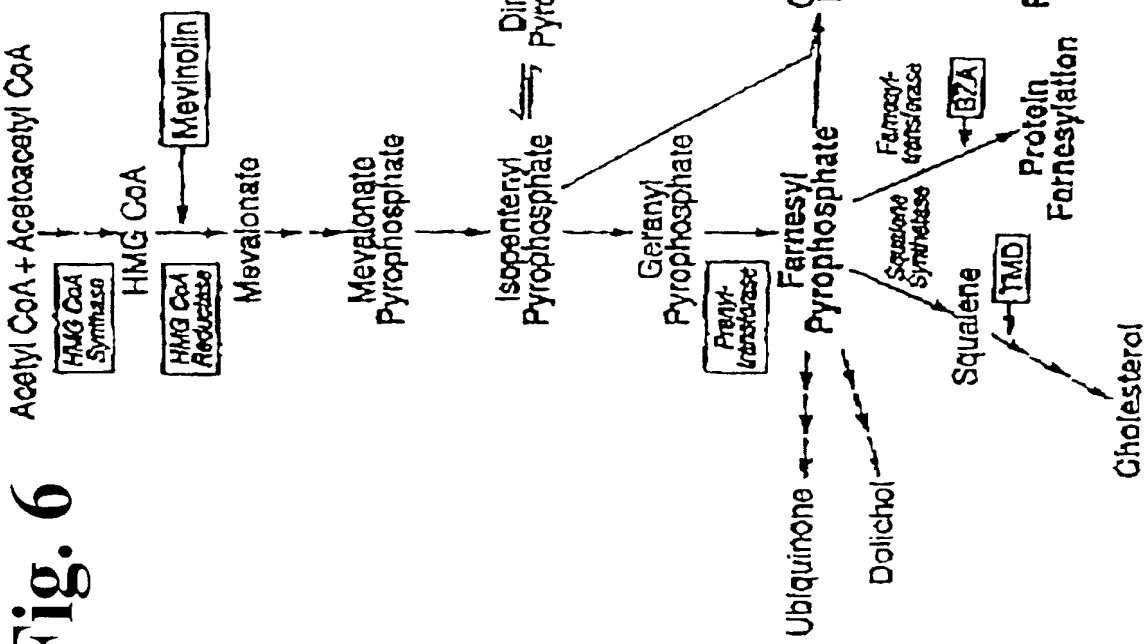


Fig.5 A-F

Fig. 6


Acetyl CoA + Acetoacetyl CoA
HMG CoA Synthase
HMG CoA
HMG CoA Reductase
Mevinolin
Mevalonate
Mevalonate Pyrophosphate
Isopentenyl Pyrophosphate
Dimethylallyl Pyrophosphate
Geranyl Pyrophosphate
Prenyl-transferase
Farnesyl Pyrophosphate
Geranyl-Geranyl Pyrophosphate
Geranyl-Geranyl transferase
Protein Geranyl-Geranylation
Farnesyl-transferase
BZA
Protein Farnesylation
Squalene Synthetase
Squalene
TMD
Cholesterol
Ubiquinone
Dolichol